REGULATION OF PROTEIN ARGININE METHYLTRANSFERASE 5 BY NOVEL SERINE 15 PHOSPHORYLATION IN COLORECTAL CANCER

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DEDICATION

This dissertation is dedicated to my Mom, Dad and all my family and friends who have shown me immeasurable love and support throughout the years.

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REGULATION OF PROTEIN ARGININE METHYLTRANSFERASE 5 BY NOVEL SERINE 15 PHOSPHORYLATION IN COLORECTAL CANCER

The overexpression of protein arginine methyltransferase 5 (PRMT5) is strongly correlated to poor clinical outcomes for colorectal cancer (CRC) patients. Previously, we demonstrated that PRMT5 overexpression could substantially augment activation of NF-κB via methylation of arginine 30 (R30) on its p65 subunit, while knockdown of PRMT5 showed the opposite effect on the transcriptional competence of p65. However, the precise mechanisms governing this PRMT5/NF-κB axis are still largely unknown. We report a novel finding that PRMT5 is phosphorylated on serine 15 (S15) in response to interleukin-1β (IL-1β) stimulation. Overexpression of the serine-to-alanine mutant of PRMT5 (S15A-PRMT5), in either HEK293 cells or HT29, DLD1 and HCT116 CRC cells attenuated NF-κB activation compared to wild type (WT)-PRMT5, confirming that S15 phosphorylation is critical for the activation of NF-κB by PRMT5. Furthermore, we found that overexpression of S15A-PRMT5 mutant attenuated the expression of a subset of NF-κB target genes through decreased p65 occupancy at their respective promoters. Importantly, the S15A-PRMT5 mutant also reduced IL-1β-induced methyltransferase activity of PRMT5 as well as its ability to form a complex with p65. Finally, we observed that the S15A-PRMT5 mutant diminished the growth, migratory and colony-forming abilities of CRC cells compared to the WT-PRMT5. Collectively, our findings provide strong evidence that novel phosphorylation of PRMT5 at S15 is critical to its regulation of NF-κB and plays an essential role in promoting the cancer-associated functions exerted by the PRMT5/NF-κB axis. Therefore, development of inhibitors to block phosphorylation of PRMT5 at S15 could become a potential novel therapeutic approach to treat CRC.

Tao Lu, Ph.D., Chair

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C. elegans	*Caenorhabditis elegans*
CARM1	coactivator Associated Arginine Methyltransferase 1
Cas9	CRISPR associated protein 9
CCL20	C-C motif chemokine ligand 20
CDK2	cyclin dependent kinase 2
CDL40	cluster of differentiation 40 ligand
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CHIP	C-terminus of Hsc70-interacting protein
COPR5	Coordinator of PRMT5 and differentiation stimulator
COX2	cyclooxygenase 2
CRC	colorectal cancer
C-terminus	carboxy terminus
CXCL10	C-X-C Motif Chemokine Ligand 10
CXCL11	C-X-C Motif Chemokine Ligand 11
3D	three dimensional
Da	daltons
DD	dimerization domain
DMEM	Dulbecco’s Modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase 3A
DSS	dextran sulfate sodium

DTT	dithiothreitol
E2F-1	E2 factor 1
EBNA-2	Epstein-Barr virus nuclear antigen 2
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
eIF4E	elongation Initiation Factor-4E
ERK	Extracellular-signal-regulated kinase 1
FBS	etal bovine serum
FDA	Food and Drug Administration
FGFR-3	fibroblast-derived Growth Factor Receptor-3
FOLFIRI	combination of 5-fluorouracil, leucovorin and irinotecan
FOLFOX	combination of 5-fluorouracil, leucovorin and oxaliplatin
5-FU	5-fluorouracil
GAPDH	glyceraldhyde 3-phosphate dehydrogenase
GAR	glycine- and arginine-rich
GRR	glycine rich regions
h	hour
H2AR3	third arginine on histone H2A
H3R2	second arginine on histone H3
H3R8	eighth arginine on histone H3
H4R3	third arginine on histone H4
HAT	histone acetyltransferase
HDAC2	histone deacetylase 2

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1α	hypoxia-inducible factor 1-alpha
HOXA9	homeobox protein Hox-A9
hSWI/SNF	Switch/Sucrose Non-Fermentable
IBD	inflammatory bowel diseases
ICAM-1	intercellular adhesion molecule 1
IκB	inhibitor of kappa B
IκBα	inhibitor of kappa B alpha
IKK	IkB kinase
IL-13	interleukin 13
IL-1β	interleukin 1 beta
IL6	interleukin 6
IL8	interleukin 8
IP	immunoprecipitation
IRES	internal ribosome entry site
IRX1	Iroquois homeobox 1
IUSM	Indiana University School of Medicine
JAK2	janus kinase 2
JBP1	JAK2 binding protein 1
KLF4	kruppel-like factor 4
KO	knockout mouse
KRAS	ki-ras2 Kirsten rat sarcoma viral oncogene homolog
KRAS	Kirsten Rat Sarcoma
LC-MS	liquid chromatography-mass spectrometry
17p LOH	short arm of chromosome 17 (17p)

18q LOH	long arm of chromosome 18 loss of heterozygosity
LPS	Lipopolysaccharide
LTβR	lymphotoxin-β receptor
m/z	mass divided by charge number
MAP3K8	mitogen-activated protein kinase kinase kinase 8
MAPK	mitogen activated protein kinase
MEP50	methylosome protein 50
mg	milligram
min	minutes
miRNA	micro RNA
MMA	x-NG-monomethylarginine
MMP2/9	matrix metalloproteinase 2/9
MMP9	matrix metallopeptidase 9
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MYC	myelocytomatosis
N	nitrogen
NAA40	N-alpha-acetyltransferase 40
NF-κB	nuclear factor kappa b
NF-YA	nuclear transcription factor Y A
NM23	nonmetastatic 23
N-terminus	amino terminus
PAFc	polymerase-associated factor complex
PBS	phosphate buffered saline
PCR	polychain reaction

PDCD4	programmed cell death protein 4
PDZ	PDZ domain
PGM	proline-, glycine- and methionine-rich
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
pICln	methylosome subunit pICln
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride
PRMT	protein arginine methyltransferase
PTM	posttranslational modification
qPCR	quantitative polymerase chain reaction
R.T.	room temperature
Rb	retinoblastoma
RHD	Rel homology domain
Riok1	RIO Kinase 1
RNA	ribonucleic acid
RNF43	ring finger protein 43
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RPS10	ribosomal protein s10
SAH	s-(5'-adenosyl)-l-homocysteine
SAM	s-adenosylmethionine
SD	standard deviation
SDMA	ω-N^G,N'^G-symmetric dimethylarginine
SDMA	symmetric x-NG, N0G-dimethylarginine

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel
SH3	SRC homology 3 domain
SEM	standard error of mean
Sin3A	paired amphipathic helix protein Sin3a
SmB	snRNP polypeptide B
SmD1	snRNP polypeptide D1
SmD3	snRNP polypeptide D3
SMN	survival motor neuron
snRNP	small nuclear ribonucleoprotein-associated protein
Sp1	specificity protein 1
SRC	sarcoma gene
SREBP1	sterol regulatory element-binding protein 1
SRR	signal responsive unit
ST7	suppressor of tumorigenicity 7
hSWI/SNF	SWItch/Sucrose Non-Fermentable
TAD	transactivation domain
TIM	triosephosphate isomerase
TNFα	tumor necrosis factor alpha
TP53	tumor protein 53
Tregs	regulatory T cells
TRP	tetratricopeptide
VCAM1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WB	western blot
Wnt	Wingless

X. laevis	*Xenopus laevis*
ZnF	Zinc Finger domain

CHAPTER 1: INTRODUCTION

1.1 Colorectal cancer

1.1.1 Risk factors, progression and common genetic causes

As the third leading cause of cancer-related deaths globally, colorectal cancer (CRC) is expected to be responsible for an estimated 1.1 million deaths by 2030 (Ferlay *et al.*, 2019). Several modifiable risk factors are attributable to a person's likelihood of developing CRC including smoking, a low-fiber high-fat diet, alcohol consumption, obesity and diabetes. Additional risk factors include increasing age, heredity conditions such as polyposis and hereditary nonpolyposis lesions as well as a history of ulcerative colitis and inflammatory bowel disease (Johnson *et al.*, 2013).

CRC patients often present with a broad spectrum of neoplasms that arise from the sequential accumulation of genetic and epigenetic aberrations (Hong, 2018). Together, these aberrations underlie the etiological basis of the progression of benign adenomas to malignant carcinomas in a well-defined model known as the adenoma-to-carcinoma sequence of CRC development (**Figure 1**) (Fearon *et al.*, 1990). Most sporadic CRCs arise through this sequence which is most commonly initiated by inactivating mutations of the adenomatous polyposis coli (APC) tumor suppressor gene during the formation of the earliest lesions known as aberrant crypt foci (L. Zhang *et al.*, 2017). Most APC mutations result in the synthesis of a truncated protein due to frameshift or nonsense mutations, which occur in approximately 30%–70% of sporadic CRCs (Schell *et al.*, 2016). As a critical component and negative regulator of the canonical Wnt signaling pathway, APC mainly exerts its tumor suppressor activity by blocking the G1 to S transition in the cell cycle thereby limiting excessive proliferation of colonic epithelial cells by inducing degradation of β-catenin (Heinen *et al.*, 2002). Deregulation of Wnt signaling and accumulation of nuclear β-catenin leads to unchecked expansion of epithelial cells (Schneikert *et al.*, 2006). This excessive proliferation is a

hallmark characteristic of early adenomas which are defined as benign tumors observed less than 1 cm in size with tubular or tubulovillous histology (Fleming *et al.*, 2012).

Activation of the oncogene KRAS signifies the second step in the progression towards advanced stages of CRC. KRAS mutations are found in approximately 50% of CRC cases and have become an important predictive marker for the effectiveness of certain treatments modalities (Andreyev *et al.*, 2001; Verdaguer *et al.*, 2017). Aberrant KRAS activation is also a hallmark of the late adenoma stage (>1 cm in size, villous histology), in which tumor cells are still confined within the epithelial layer but tend to show disorganized growth and develop into highly dysplastic tumors. The most commonly observed mutations for KRAS are substitutions at codons 12 and 13 (G12D, G13D, respectively) both located in exon 2 and occur in nearly 60% of colon ademonas (Jones *et al.*, 2017). These activating mutations are an integral step in achieving constitutive activation of the mitogen activated protein kinase (MAPK) pathway which leads to unchecked proliferation, survival, angiogenesis, invasion, and metastasis of malignant colon epithelial cells via upregulation of associated target gene products (Hatzivassiliou *et al.*, 2013). Sustained activation of MAPK signaling may also occur via a V600E point mutation in BRAF which leads to constitutive activation (Morkel *et al.*, 2015).

Further malignant transition towards the carcinoma stage is accompanied by loss of heterozygosity (LOH) of the long arm of chromosome 18 (18q) and short arm of chromosome 17 (17p) (Pino *et al.*, 2010). 18q/17p LOH, along with concomitant mutational inactivation of the tumor suppressor TP53 gene, constitutes a critical final step in the development of malignant colorectal carcinomas (C.-Z. Zhou *et al.*, 2004). These tumors are characterized by their ability to invade the surrounding tissues and eventually metastasize to distal organs such as the liver. Additionally, several studies have endeavored to elucidate the prognostic value of the TP53 mutation status by

demonstrating a link between these mutations and lymphatic invasion in proximal CRC (Russo *et al.*, 2005). Patients with mutant TP53 also appear to be more chemo-resistant with poorer prognosis compared to those with wild-type TP53 (Iacopetta, 2003).

1.1.2 Epigenetic causes

The development of CRC has also been linked to the accumulation of multiple epigenetic alterations (Baretti *et al.*, 2018). Unlike genetic mutations, epigenetic modifications consist of heritable changes in gene expression without DNA sequence changes and are intrinsically reversible by nature. Moreover, the reversibility of these modifications makes them attractive molecular targets for anticancer therapeutic interventions (Kelly *et al.*, 2010; Okugawa *et al.*, 2015). Assessment of the epigenetic landscape of CRC reveals that virtually all cases harbor abnormal changes in DNA methylation and histone modifications (Irizarry *et al.*, 2009). DNA methylation for instance, constitutes one of the first recognized epigenetic alterations in CRC, where global DNA hypomethylation and discrete, systematic hypermethyation are frequently observed. More precisely, global DNA hypomethylation, which mainly takes place on cytosine guanine (CpG) dinucleotides within pericentromeric regions, is primarily associated with widespread oncogene activation and increased genomic instability. Discrete DNA hypermethylation however, typically occurs at CpG dinucleotide-dense regions, called CpG islands. These CpG islands are found within the promoters of many tumor suppressor genes, resulting in transcriptional silencing (e.g., APC). However, other reports have shown that this hypermethylation also exists within the first exonic/intronic regions of several other genes involved in DNA repair, apoptosis, proliferation, angiogenesis, adhesion, and invasion [e.g., Secreted frizzled related protein 5 (SFRP5), Insulin-like growth factor 2 (IGF2) and Cyclin-dependent kinase inhibitor 2A (p16INK4a/CDKN2A)] (Irizarry *et al.*, 2009; Jia *et al.*, 2013). Many of these

changes occur gradually and begin early in the process of colorectal carcinogenesis (Luo *et al.*, 2014).

Furthermore, abnormal histone methylation and acetylation have been shown to be highly recurrent and serve as important biomarkers to predict clinical outcome for CRC patients (Vaiopoulos *et al.*, 2014). For instance, reduced levels of H3K9me3 and H4K20me3 found within circulating nucleosomes are strongly correlated with poor CRC patient outcome. Conversely, high H4K20me3 and H3K9me3 but low nuclear expression of H3K4me3 are usually associated with a better prognosis for early-stage CRC patients (Benard *et al.*, 2014; Tamagawa *et al.*, 2012). Interestingly, many of these post-translational marks coincide with repression and activation of key tumor suppressors and oncogenes, respectively (T. Huang *et al.*, 2017; Kodach *et al.*, 2010). For example, DNA hypermethylation-mediated silencing of several Wnt pathway inhibitors (e.g., AXIN1, AXIN2, WTX, RNF43) has been suggested as a main contributor to hyperactivation of the Wnt pathway (Galamb *et al.*, 2016). Thus, epigenetic regulation represents another critical mechanism by which cancer cells achieve tumor-promoting transcriptional changes in the absence of genetic mutations. With such profound regulatory potential, it is not surprising that deregulation of certain key epigenetic modulators gives rise to many of the malignant processes commonly seen in CRC (T. Huang *et al.*, 2017). Indeed, changes in the expression or activity levels of several classes of epigenetic modulators have been linked to CRC, particularly methyltransferases and histone deacetylases (Vaiopoulos *et al.*, 2014).

These studies collectively highlight the complexity and heterogeneity of CRC, further underscoring the need to identify new biomarkers and therapeutic targets for improved clinical management of this challenging disease.

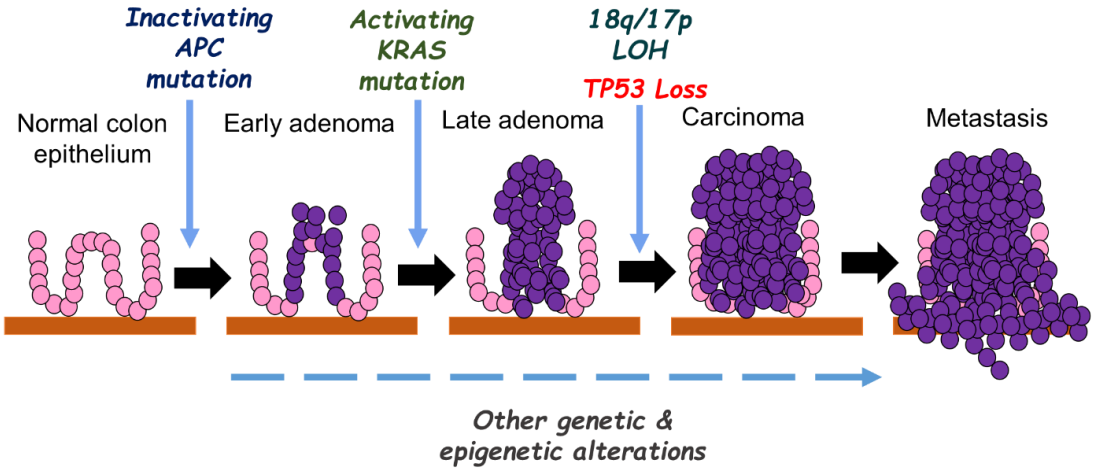


Figure 1: Schematic of the adenoma-to-carcinoma sequence of CRC (Walther *et al.*, 2009)**.** The temporal order in which changes in key genes may affect the progression of CRC is shown above the histological stages of disease. Briefly, this sequence is initiated by inactivating mutations of the *APC* tumor suppressor gene during the formation of the earliest lesions, aberrant crypt foci (ACF; not shown). These ACFs evolve into early adenomas which are defined as benign growths less than 1 cm in size with tubular or tubulovillous histology. Transition to a late adenoma stage is achieved by activating mutations in the *KRAS* oncogene. Late adenomas are greater than 1 cm in size, still confined within the epithelial layer and tend to show disorganized growth and dysplasia. Finally, loss of heterogeneity (LOH) of the long arm of chromosome 18q and short arm of chromosome 17p as well as inactivating mutations of the TP53 gene increase chromosomal instability, which are crucial events in the transition of tumors cells into carcinomas and metastases (capable of invading the surrounding tissues).

1.1.3 Current treatment options and limitations

With important advances in early screening, the 5-year survival rate (≥60%) for patients diagnosed at early stages of CRC has made tremendous leaps over the years (Simon, 2016). For these patients, surgery along with adjuvant chemotherapy are the mainstay treatment for resectable tumors. Unfortunately, more than half of patients present with metastatic CRC (mCRC) at the time of diagnosis in which case the outlook is dismal with a 5-year survival rate of only 10% (Nakayama *et al.*, 2013). For patients with mCRC, surgery is usually not an option and chemotherapeutic intervention is the only means of improved survival. Before the advent of combinatorial chemotherapeutic regimens, the standard of care for unresectable mCRC was first-line monotherapy with 5-fluorouracil, a cytotoxic agent that exerts its anticancer effects by blocking DNA synthesis through inhibition of thymidylate synthase. Today, combination regimens such as FOLFIRI (combination of 5-FU, leucovorin, and irinotecan) and FOLFOX (combination of 5-FU, leucovorin, and oxaliplatin) have now become the cornerstone of anti-cancer therapy (Benson *et al.*, 2013). The use of targeted therapies has also become increasingly important and several monoclonal antibodies against vascular endothelial growth factor (VEGF; bevacizumab) and epidermal growth factor receptor (EGFR; cetuximab) have been incorporated into the routine clinical care of mCRC patients (Saltz *et al.*, 2008; Van Cutsem *et al.*, 2011). Notably, the mutational status of KRAS is a major predictor of resistance to cetuximab therapy where patients with mutated KRAS (G12D) have a worse prognosis (Arrington *et al.*, 2012). Another targeted agent which is used after other lines of therapy have failed is regorafenib, a multikinase inhibitor that affects several signaling pathways including VEGF signaling (Goel, 2018).

With the growing global burden of a high incidence rate, prevention and treatment of CRC remains a significant public health challenge. Unfortunately, due to the extreme aggressiveness and complex molecular heterogeneity of these tumors as well

the emergence of therapeutic resistance, the afore-mentioned treatment modalities are hardly curative, necessitating treatment for many years (Cidón, 2010). Moreover, progress in developing new treatment options for CRC has been relatively static over the past decade, underscoring the urgent need to better understand molecular mechanisms that drive CRC which can be translated into clinical utility.

The nuclear factor-kappa B (NF-κB) signaling pathway serves as a central coordinator of immune and inflammatory responses and has been implicated in driving several hallmark tumorigenic processes (T. Liu *et al.*, 2017; Xia *et al.*, 2014). Importantly, constitutive activation of NF-κB is also frequently observed in CRC and has garnered increasing attention as a potential therapeutic target (Vaiopoulos *et al.*, 2013). The following sections discuss the current evidence linking NF-κB hyperactivity to CRC initiation and progression, lending credence to targeting this important pathway as a promising treatment strategy for CRC.

1.2 NF-κB signaling and its role in CRC

1.2.1 Overview of NF-κB signaling pathways

The family of NF-κB transcription factors consists of five members, namely, p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2). These proteins form distinct homo- or heterodimeric complexes, with the p65/p50 heterodimer being the most prominent (Dolcet *et al.*, 2005). Notably, both p50 and p52 are produced by proteasomal processing of their precursors p105 and p100, respectively. Although structurally diverse, all NF-κB family members share a highly conserved domain – the N-terminal Rel homology domain (RHD), which is required for dimerization, DNA binding, interaction with the inhibitors of NF-κB (IκBs) and nuclear translocation (Karin *et al.*, 2000). By contrast, the C-terminal transactivation domain (TAD) is conserved only among the Rel proteins, including p65 (RelA), RelB, and c-Rel and confers

transcriptional competence (Karin *et al.*, 2000). In unstimulated cells, NF-κB dimers are latent and sequestered in the cytoplasm via association with the inhibitory IκB family of proteins. The IκB family also consists of several members (IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100, and p105) with IκBα and IκBβ being known for their prominent roles in binding to NF-κB heterodimers, effectively blocking their nuclear localization. All members of the inhibitory IκB complexes including p100 and p105 are characterized by the presence of ankyrin repeat domains (ANK) in their structure, which are purported to mask the nuclear localization signals (NLS) of NF-κB heterodimers, thus sequestering them in the cytoplasm (Karin *et al.*, 2000). A detailed depiction of the domain architecture of various NF-κB family members is shown in **Figure 2**.

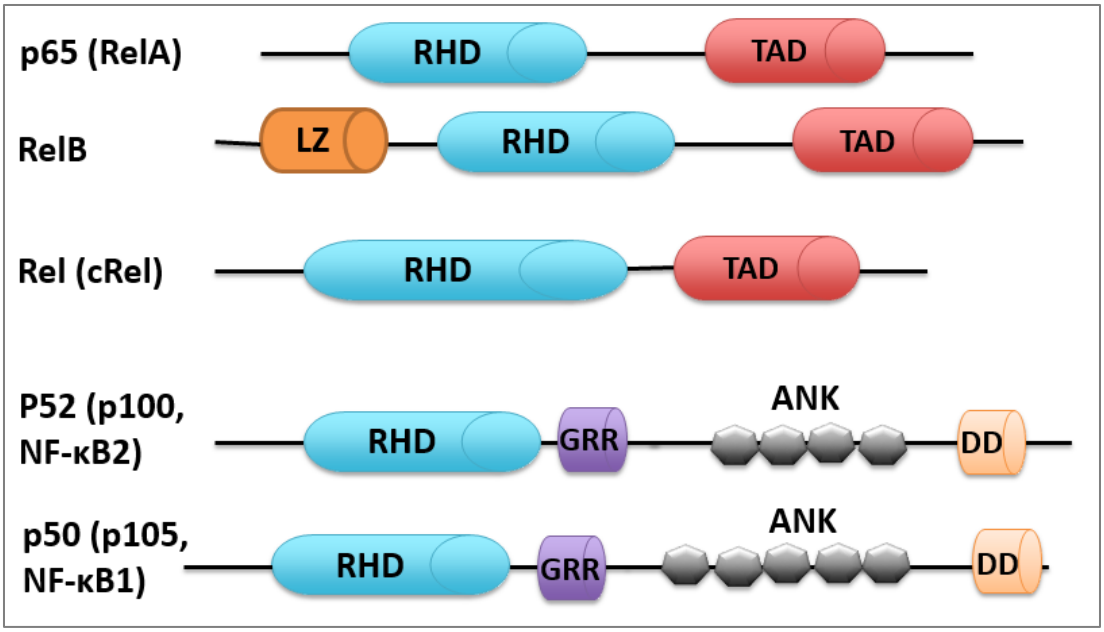


Figure 2: Schematic of the NF-κB family members (Hartley *et al.*, 2018). The NF-κB family members are defined by the N-terminal Rel Homology Domain (RHD), which is responsible for DNA binding and dimerization. All except p52 and p50 contain a Transactivation Domain (TAD), which confers positive regulation of gene expression. p52 and p50 also contain glycine rich regions (GRR), which are necessary for their proteolytic cleavage and ankyrin repeats (ANK) similar to those found in IκB family of inhibitor proteins. Additionally, RelB contains a leucine zipper motif (LZ). Other abbreviation: DD, dimerization domain. Permission to use all or part of this published figure is outlined in **Appendix A**.

Activation of NF-κB can be classified into two distinct pathways, commonly referred to as the canonical and non-canonical pathways (**Figure 3**). In both pathways, NF-κB heterodimers are retained in the cytoplasm by IκB proteins under resting states. In general, NF-κB can be activated by a diverse array of stimuli that lead to IκB kinase (IKK)-dependent phosphorylation, polyubiquitination, and subsequent proteasome-mediated degradation of IκB proteins, a common regulatory step for both pathways. The liberation of NF-κB subunits then allows them to translocate to the nucleus, where they can bind to cognate κB sites in specific promoter regions to activate target gene expression (Shih *et al.*, 2011; S.-C. Sun, 2017).

The canonical pathway, which primarily regulates the transcription of genes involved in inflammation, innate immunity and cell survival, is typically simulated by factors such as tumor necrosis factor α (TNFα), interleukin 1 (IL-1), or lipopolysaccharide (LPS), leading to IKK activation. The IKKβ subunit of the IKK complex phosphorylates serine residues in the signal responsive region (SRR) of IκBα, leading to its ubiquitination and subsequent proteasomal degradation. No longer sequestered by IκBα, the p65/p50 heterodimer is now free to translocate to the nucleus to induce transcription of target genes (Greten & Karin, 2004; Hayden *et al.*, 2004). By contrast, the non-canonical pathway, which is mainly implicated in the regulation of B-cell maturation, humoral immunity and lymphoid organ development, depends on NF-κB-inducing kinase (NIK)-induced activation of IKKα and is typically simulated by ligands such as cluster of differentiation 40 ligand (CD40L), LPS, and B-cell activating factor (BAFF). In this pathway, p100/RelB complexes are retained in an inactive state in the cytoplasm. Signaling through a small subset of receptors such as lymphotoxin-β receptor (LTβR), CD40, and BAFF receptor 3 (BR3) activates NIK which is rapidly recruited to phosphorylate IKKα. The net effect is phosphorylation and ubiquitination of p100, and its subsequent proteasomal processing to p52. This signaling cascade creates a

transcriptionally competent RelB/p52 complex that can translocate to the nucleus and initiate target gene expression (Bonizzi *et al.*, 2004; S.-C. Sun, 2017).

Finally, although abnormal activation of both arms of this signaling pathway is involved in tumorigenesis and is known to play critical roles in tumor growth, progression, and therapeutic resistance, the canonical NF-κB pathway has been more extensively studied and implicated in solid tumors such as CRC (Hoesel *et al.*, 2013). On the other hand, the non-canonical pathway has been documented as primarily playing a role in hematological malignancies such as multiple myeloma (Imbert *et al.*, 2017). Since CRC is the main cancer of interest in this study, discussion in the subsequent sections will be in reference to canonical NF-κB signaling.

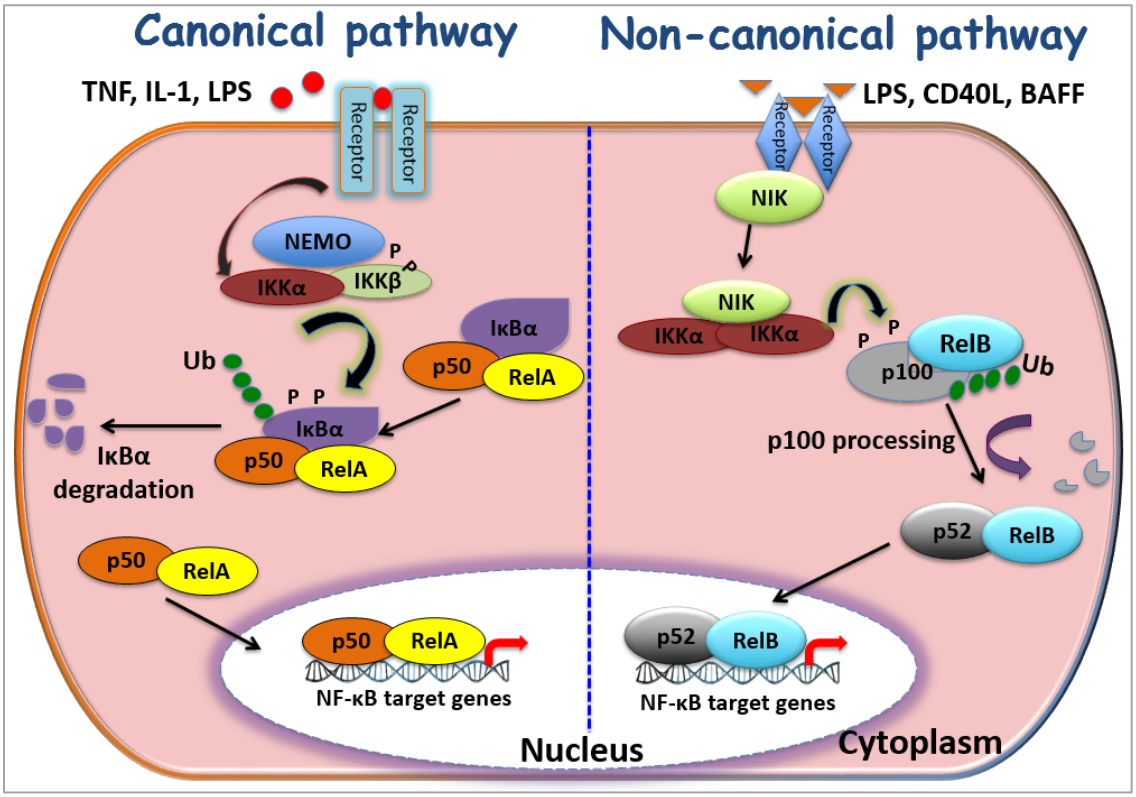


Figure 3: Schematic of the canonical and non-canonical NF-κB pathways (Hartley *et al.,* 2018). The canonical pathway (left) is induced by most physiological NF-κB stimuli and is represented here by TNF, IL-1 and LPS signaling. Stimulation of the corresponding receptor leads to the IKK complex activation comprised of two catalytic subunits, IKKα and IKKβ as well as the regulatory IKKγ or NEMO subunit. IκBα is then phosphorylated in an IKKβ- and NEMO-dependent manner, which results in its polyubiquitination and subsequent degradation. The liberated p65/p50 heterodimer undergoes nuclear translocation where it engages in target gene transcriptional activation. The non-canonical pathway (right) is induced by a more selective family of molecules, such as LPS, CD40L, BAFF and lymphotoxin-β (LT-β). Upon activation, p100 processing depends on NIK, which triggers IKKα-mediated phosphorylation of p100, leading to partial processing of p100 and the generation of transcriptionally active p52/RelB complexes. Permission to use all or part of this published figure is outlined in **Appendix A**.

1.2.2 NF-κB and IL-1β signaling in CRC initiation and progression

Over the past decade, NF-κB has emerged as a master regulator of inflammatory responses and tumor development. In CRC especially, NF-κB has been shown to be constitutively activated in nearly 60–80% of patient tumors (S. Wang *et al.*, 2009). Constitutive NF-κB activity seems to play a critical role in virtually all the hallmarks of cancer and contributes to both tumor initiation and progression. This is largely accomplished by its upregulation of the expression of a diverse array of target genes, many of which mediate malignant processes such as cellular proliferation (e.g., cyclin D1), anti-apoptosis [(e.g., survivin, B-cell lymphoma 2 (Bcl-2), X-linked inhibitor of apoptosis protein (XIAP), inhibitor of apoptosis protein 1 (IAP1)], angiogenesis [(e.g., vascular endothelial growth factor (VEGF), interleukin-8 (IL-8), cyclooxygenase 2 (COX2)], and metastasis [(e.g., matrix metallopeptidase 9 (MMP9), intercellular adhesion molecule 1 (ICAM-1)] (Hassanzadeh, 2011).

Furthermore, a wealth of evidence supports the role of NF-κB-mediated chronic inflammation as one of the key etiologic factors in CRC development. In fact, a classic example is inflammatory bowel diseases (IBDs), in which constitutive NF-κB activation has been shown to significantly increase the risk of CRC development in patients with a number of years of active disease (Atreya *et al.*, 2008; Eaden *et al.*, 2001). The interconnection between inflammation and cancer was initially proposed by Virchow in the mid-nineteenth century when he hypothesized that cancer arose at regions of chronic inflammation brought about by irritants and tissue injury (Balkwill *et al.*, 2001). IBD is associated with persistent NF-κB activation in cells such as the myeloid and epithelial cells located within the colonic mucosa. This results in a sustained inflammatory process in the gut mucosa that promotes cell survival and tumorigenesis (Greten, Eckmann*, et al.*, 2004). As such, aberrant NF-κB activation was shown to bring

about the malignant transformation of colon epithelial cells via its transcriptional upregulation of pro-inflammatory cytokines, chemokines and growth factors (Neurath *et al.*, 1998). Once these inflammation-driven sporadic tumors are formed, elevated NF-κB activity is further achieved by the induction of a local network of cytokines/chemokines and immune/inflammatory cell infiltration into affected sites (Greten, Eckmann*, et al.*, 2004). Among these cytokines and chemokines, are a host of NF-κB target genes such as TNF-α and IL8, the upregulation of which has been shown to strongly correlate with CRC severity and poor prognosis (Mager *et al.*, 2016). The continuous release of cytokines and growth factors by both tumor and immune cells within the tumor microenvironment results in a feed-forward, prolonged retention of nuclear NF-κB. Ultimately, this sustained NF-κB-mediated inflammation stimulates tumor proliferation and invasiveness, amplifying the overall metastatic potential of CRC cells (**Figure 4**) (Cooks *et al.*, 2013). As expected, inhibition of the NF-κB pathway and subsequent downregulation of relevant target genes has been shown to attenuate the formation of tumors in a CRC mouse model (Greten, Eckmann*, et al.*, 2004).

In addition to its pro-inflammatory role, NF-κB has also been widely implicated in mediating chemoresistance through the activation of a multitude of anti-apoptotic and pro-survival target genes (Godwin *et al.*, 2013). For instance, several studies have shown that aberrant activation of NF-κB mediates 5-FU resistance in CRC cells, which constitutes one major reason for the failure of mCRC treatment (Konishi *et al.*, 2006; Z. Wang *et al.*, 2018). Unsurprisingly, NF-κB inhibitors such as resveratrol, SN50 and quinacrine which block the transcriptional activity, nuclear translocation and DNA binding of p65, respectively, when used in combination with chemotherapeutics 5-fluorouracil and oxaliplatin, have been shown to synergistically mitigate CRC cell growth (Buhrmann *et al.*, 2018; Jani *et al.*, 2010; Z. Wang *et al.*, 2018). In another study, combination of irinotecan with NF-κB-targeting thymoquinone was shown to increase sensitivity of CRC

cells to irinotecan (M. C. Chen *et al.*, 2017). Taken together, these findings implicate NF-κB as an important therapeutic target in CRC.

IL-1β is a pleiotropic pro-inflammatory cytokine abundantly secreted at tumor sites primarily by epithelial and inflammatory cells, mediating the induction of a local network of cytokines/chemokines and cell infiltration into affected sites (Apte *et al.*, 2006). This in turn significantly affects the process of tumorigenesis by modulating tumor growth and invasiveness, tumor-mediated angiogenesis, and also the patterns of tumor-host interactions in the reactive tumor microenvironment (Apte *et al.*, 2006; Carmi *et al.*, 2013). Importantly, a high IL-1β concentration within the tumor microenvironment has been associated with a more malignant tumor phenotype and in CRC, is shown to promote the invasiveness of colon tumor cells (Li *et al.*, 2012). The tumor-promoting role of IL-1β in the cancer microenvironment is further augmented by the fact that it serves as a potent inducer of NF-κB activity (Hai Ping *et al.*, 2016). In the proposed work, we have established a critical link between IL-1β-mediated PRMT5 phosphorylation and enhanced NF-κB activation, which is of potentially enormous biological significance. We propose that activation of this PRMT5/NF-κB signaling node by IL-1β secreted by cells in the tumor microenvironment could concertedly promote inflammation-associated tumor invasiveness. This is an exciting possibility to explore in the near future.

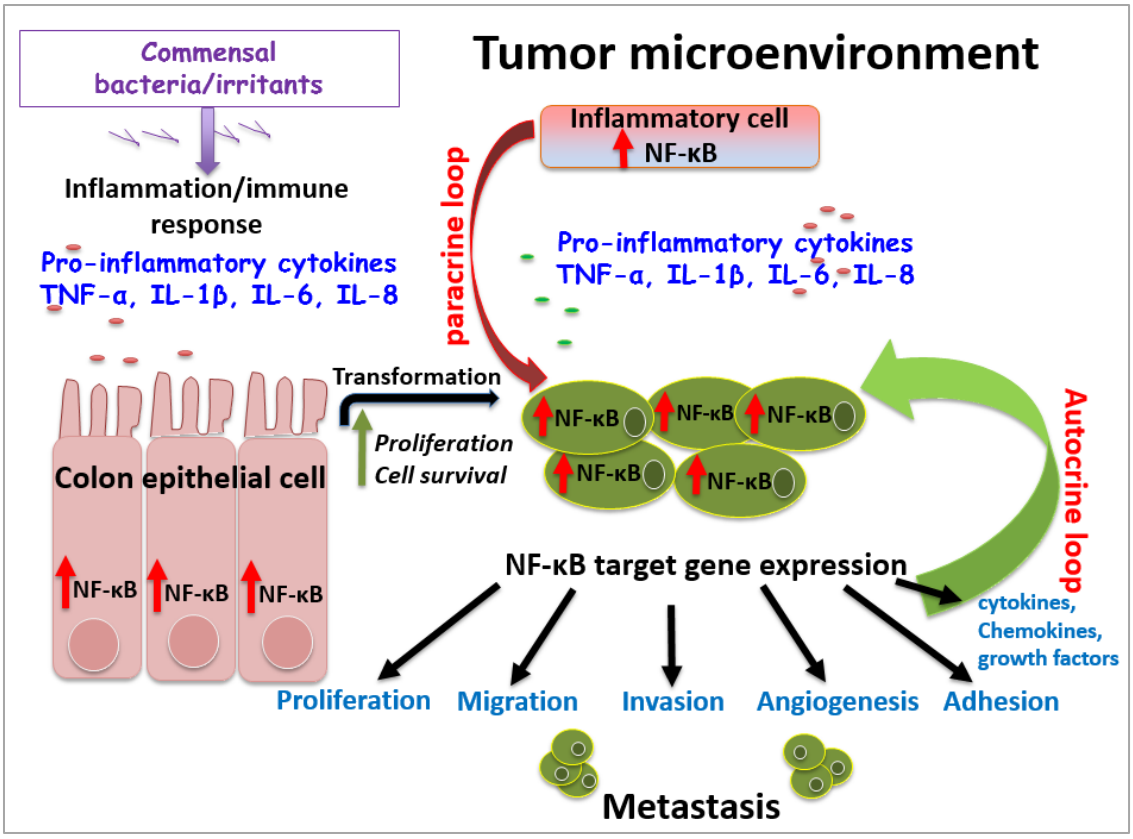


Figure 4: Constitutive activation of NF-κB promotes inflammation and CRC progression (Hartley *et al*., 2018). Bacteria and various "irritants" of the gut cause tissue injury and inflammation, leading to excessive production of pro-inflammatory cytokines. These cytokines bind to cell surface receptors of colon epithelial cells, resulting in NF-κB activation in these cells. NF-κB activation in colon epithelial cells increases cell proliferation and survival via upregulation of proliferative and pro-survival NF-κB target genes, thus contributing to the malignant transformation of these cells. Activation of NF-κB in inflammatory cells from the tumor microenvironment also contributes to CRC development by inducing expression of cytokines, chemokines and growth factors. This leads to constitutive activation of NF-κB in tumor cells, which in turn, release numerous factors that sustain the ongoing paracrine inflammatory process between the tumor microenvironment and tumor cells and the autocrine loop between tumor cells. Hyperactive NF-κB in tumor cells promotes expression of diverse NF-κB target genes, including cytokines, chemokines and growth factors, as well as genes that are associated with metastasis, such as those promoting proliferation, migration, angiogenesis, adhesion, and invasion of tumor cells. Permission to use all or part of this published figure is outlined in **Appendix A**.

1.2.3 Limitations of current NF-κB inhibitors

Because of the multitude of cancer-initiating and malignant processes affected by NF-κB signaling, much effort has been made by the pharmaceutical industry to develop inhibitors to target this pathway. To date, over 750 inhibitors have been identified, ranging from small molecules, natural compounds and their derivatives to small DNA/RNAs, engineered polypeptides and viral proteins (Gilmore *et al.*, 2006). Notably, development of several of these inhibitors is targeted towards impeding one or several nodes along the canonical NF-κB signaling pathway. For instance, the development of inhibitors based on blockade of IKK or proteasomal degradation of IκBα has been amply described (Llona-Minguez *et al.*, 2013). Certain phytochemicals such as curcumin, green tea extract, ginseng and resveratrol have been shown to inhibit IKK activity (Gonzales *et al.*, 2008; Gupta *et al.*, 2010; Su *et al.*, 2006). Proteasome inhibitors (e.g., bortezomib), which have generally yielded more success, act by blocking the degradation of IκBα to enhance cytoplasmic retention of NF-κB heterodimers (Adams, 2002). However, very few documented reports exist to support their efficacy in triggering apoptosis when used as a monotherapy in cancer (Hartley *et al*., 2018).

Unfortunately, the vast majority of these inhibitors have not yet been approved for clinical applications in cancer. Moreover, several molecules including non-steroidal anti-inflammatory drugs (NSAIDs) were found to only collaterally limit NF-κB activity and thus lack selectivity for cancer cells. Besides, there is a certain degree of reluctance associated with direct and prolonged inhibition of NF-κB, which is inherently complicated and challenging on many levels. First, NF-κB cooperates with a multitude of other signaling molecules and pathways, many of which directly interact with NF-κB subunits or affect NF-κB target genes in normal cellular processes. These molecules may or may not themselves be contributors to the cancer process and thus, disruption of these critical signaling nodes of crosstalk using broad-spectrum inhibitors could potentially

yield undesirable secondary effects. Second, NF-κB is also an essential player in the immune response against cancer. Thus, global and prolonged immunosuppression via direct or nonspecific NF-κB inhibition is likely to have deleterious effects on patients due to associated immune-related toxicities (Baud *et al.*, 2009). To further complicate matters, some standard chemotherapeutic agents such as doxorubicin, irinotecan and 5-FU can inadvertently activate the NF-κB pathway via induction of proinflammatory cytokines (eg IL-1β, TNFα) and cellular stressors (e.g., ROS) or by activating DNA-repair mechanisms (Bednarski *et al.*, 2008; W. Wang *et al.*, 2017). Theoretically, an ideal NF-κB inhibitor should therefore prevent NF-κB activation and be more selective in destroying malignant cells with little to no adverse effects on other signaling pathways or interference with NF-κB's physiological roles in immunity, inflammation, and cellular homeostasis.

Although we may be a long way from developing the most effective and least toxic anti-NF-κB agent, a more indirect yet cancer-selective inhibition of NF-κB signaling may be achieved by first identifying key proteins that are frequently deregulated in cancer and are known to activate this pathway in tumor cells. These proteins can then be selectively targeted in cancer cells in lieu of broad-spectrum inhibition of NF-κB, given its pleiotropic and ubiquitous functions. This approach has been used with some success which involves the use of inhibitors against upstream regulators of NF-κB, such as PI3K and mTOR (Ahmad *et al.*, 2013). In this respect, we previously established a connection between overexpression of PRMT5 which is frequently observed in CRC, and enhanced activation of NF-κB and its target genes (Prabhu *et al.*, 2017; Wei *et al.*, 2013). Importantly, the work described in this thesis demonstrates a novel phospho-dependent relationship between PRMT5 and NF-κB in CRC. Disruption of the PRMT5/NF-κB signaling axis by blocking phosphorylation of PRMT5 may thus serve to broaden options for therapeutic intervention. The next sections provide an overview of

PRMT5, including its general biological roles, specific contribution to cancer as well as insights into the regulation of its activity.

1.3 PRMT5

1.3.1 Overview of PRMT superfamily members and arginine methylation

Post-translational modifications (PTMs) have evolved as focal points of signal transduction. They are crucial to initiating and augmenting signaling cascades by fine-tuning the function of the molecules they alter (Deribe *et al.*, 2010). Moreover, many PTM events may in part underlie the transcriptional and epigenetic-driven changes in gene expression that lie at the heart of various normal cellular requirements. Unsurprisingly, in pathological states, the enzymes that catalyze the addition and removal of PTMs are frequently dysregulated and have emerged as major drug targets for a variety of diseases, including cancer.

Arginine methylation continues to garner intense interest as a key PTM, largely due to its role as a regulator of both histone and non-histone proteins involved in diverse cellular processes. These processes include splicing, RNA processing, transcription, development, DNA damage repair, proliferation, and signal transduction (Bedford *et al.*, 2009). The result of arginine methylation is the addition of methyl groups to the guanidino nitrogen atoms of arginine residues in proteins; the complexity is further augmented by the addition of either one or two methyl groups. Specifically, there are three main types of arginine methyl marks: ω-N^G, monomethylarginines (MMA); ω-N^G,N^G-asymmetric dimethylarginines (ADMA); and ω-N^G,N'^G-symmetric dimethylarginines (SDMA) (Bedford *et al.*, 2009). These marks are deposited by the family of protein arginine methyltransferases (PRMTs) that consists of nine members, classified by which mark they catalyze. For instance, type I, II and III PRMTs all catalyze the formation of MMA intermediates, while type I PRMTs (PRMT1, 2, 3, 4, 6 and 8) also

catalyze the production of ADMA. Meanwhile, type II PRMTs (PRMT5 and PRMT9) catalyze the formation of SDMA. PRMT7 was described as exhibiting type III enzymatic activity due to its preferential formation of MMA on histones, its only known substrates (**Figure 5**) (Zurita-Lopez *et al.*, 2012). More recently, a type IV enzyme which remains to be fully characterized was identified only in yeast as monomethylating the internal (or δ) guanidino nitrogen atom. Notably, however, no homolog of this enzyme has been identified in higher eukaryotic organisms (Zobel-Thropp *et al.*, 1998).

The majority of PRMT substrates harbor conserved glycine (G)- and R-rich (GAR) motifs. A notable exception is PRMT4 (CARM1) which methylates arginine residues within proline and glycine-rich (PGM) motifs (Bedford *et al.*, 2009; Gayatri *et al.*, 2016). Others such as PRMT5, can methylate arginine residues located within both GAR and PGM motifs (Branscombe *et al.*, 2001; Cheng *et al.*, 2005). In addition to these substrate recognition motifs, the nine members of human PRMT family share a highly homologous S-adenosylmethionine (SAM)-dependent methyltransferase domain known as the Rossman fold, which serves as the catalytic core. However, the human PRMT family members also have divergent structural motifs (**Figure 6**). For instance, PRMT2, PRMT3, PRMT4, PRMT5 and PRMT9 all have N-terminal motifs preceding the catalytic domain. In PRMT2 and 3, a SRC Homology 3 Domain (SH3) domain and zinc-finger domain (ZnF) precedes the Rossman fold, respectively. Meanwhile, a pleckstrin homology (PH), triosephosphateisomerase (TIM) barrel and tetratricopeptide (TRP) motif precedes the catalytic domain in PRMT4, 5 and 9, respectively (Cheng *et al.*, 2005; Schapira *et al.*, 2014). Generally speaking, these additional N-terminal motifs have been purported to regulate PRMTs in terms of their substrate recognition or complex formation and thus serve to contribute to their nonredundant roles in different biological processes (Stephen Antonysamy *et al.*, 2012; Kozbial *et al.*, 2005).

Although MMA, ADMA and SDMA all potentially have different functional consequences, the overall main effect of arginine methylation is alteration of protein-protein, protein-DNA, and protein-RNA interactions by the methylated protein (Dolzhanskaya *et al.*, 2006). This is evidenced in the structural effects conferred by this PTM. For example, each arginine residue of a protein has five potential hydrogen-bond donors which interact with the hydrogen-bond acceptors of its interacting partners. The addition of a methyl group therefore sterically affects these interactions by providing increased hydrophobicity without changing the overall charge of the residue. Furthermore, methylation adds to the overall mass of the protein as indicated by a mass shift of ~14 Da (MMA) or ~28 Da (ADMA or SDMA). Ultimately, this results in either positive or negative effects on the interaction of the methylated proteins with other molecules (Blanc *et al.*, 2017).

Interestingly, it also not uncommon for type I and II PRMTs to share the same substrates with differential outcomes. For example, a recent study from our lab demonstrated that PRMT5-mediated symmetric methylation of p65 at R30 (R30me2s) positively regulated the DNA-binding ability of p65 and its capacity to drive transcriptional activation of target genes (Wei *et al.*, 2013). Moreover, predicted modelling revealed a stabilizing effect of R30me2 through van der Waals contacts that indirectly increased the affinity of p65 for DNA. Conversely, Reintjes *et al* (2016) reported a mechanism by which TNFα-induced asymmetric dimethylation of p65 by PRMT1 (R30me2a) resulted in impaired binding of NF-κB to gene promoters (Reintjes *et al.*, 2016). Thus, in this case, ADMA and SDMA marks on p65 may potentially represent a specific on/off switch mechanism for moderating cytokine-induced NF-κB responses. Intriguingly, a similar phenomenon was observed with another transcription factor, E2F-1, whereby competitive PRMT5- and PRMT1-catalyzed SDMA and ADMA marks, respectively had differential effects on its DNA-binding and transactivating potential (S.

Zheng *et al.*, 2013). This dichotomy suggests a nuanced and likely context-specific structural effect of ADMA versus SDMA moieties on a given substrate protein. Arginine methylation has also been shown to affect other critical aspects of a protein, including its stability, subcellular localization and enzymatic activity. For instance, methylation of CARM1-mediated methylation of SRC-3 was shown to enhance its turnover whereas methylation of RNA helicase A is required for its nuclear import (Naeem *et al.*, 2007; Smith *et al.*, 2004). Undoubtedly, the above findings are only a fraction of the roles of arginine methylation in diversifying the functions of the proteome. It is possible that even more roles will be uncovered in the near future, particularly as it relates to the involvement of this PTM in the pathogenesis of various diseases, including cancer.

1.3.2 PRMT5: Biological roles and contribution to cancer

PRMT5 is the main type II methyltransferase and is responsible for 95 percent of the symmetric dimethylation observed in cells. In addition to histones, PRMT5 methylates a wide variety of non-histone substrates such as Sm proteins, transcription factors (e.g., p65, p53) and signaling effector proteins (e.g., RAF). Importantly, these methylation events impact several biological processes, including transcriptional control, proliferation, DNA damage response and repair, splicing, signal transduction, differentiation and development, among others (Stopa *et al.*, 2015).

1.3.2A General biological roles of PRMT5

Before its methyltransferase activity was biochemically characterized, PRMT5 was first discovered as a Janus kinase 2 (JAK2) binding protein and initially named JBP1 (Pollack *et al.*, 1999). JBP1 has since been established as the main type II PRMT, playing multiple roles in a wide range of cellular processes (Stopa *et al.*, 2015). PRMT5 is ubiquitously expressed and its catalytic domain is highly conserved across different

species. Importantly, its depletion during development results in early embryonic lethality (Tee *et al.*, 2010). This loss of viability is attributed to the genomic instability accrued during global DNA demethylation, leaving blastocysts vulnerable to the transcriptional activation of harmful transposable elements (Kim *et al.*, 2014). Another group demonstrated that conditional knockout of PRMT5 in neural stem/progenitor cells (NPCs) resulted in postnatal death in mice (Bezzi *et al.*, 2013). Together, these findings suggest the indispensable role of PRMT5 in development, which is exerted by its methylation of a diverse array of histone and non-histone substrates. An illustrative list of known PRMT5 substrates is provided in **Table 1**.

In addition to its association with developmental pathways, one of the primary functions of PRMT5 involves its extensive role in transcriptional regulation. This occurs most notably through methylation of histones or modulation of the activity of transcription factors. Moreover, several studies suggest that PRMT5 acts either as an activator or repressor of transcription depending on the substrate it modifies as well as the chromatin context. For instance, during the early stages of development, PRMT5 dimethylates H2AR3 (H2AR3me2s) which is critical for maintaining embryonic stem cell pluripotency through repression of differentiation genes (Gkountela *et al.*, 2014; Vougiouklakis *et al.*, 2018). Others have shown that PRMT5 also associates with heterochromatin in erythroid progenitor cells, where it symmetrically dimethylates H4R3 (H4R3me2s) resulting in the recruitment of co-repressors to further potentiate gene repression (Zhao *et al.*, 2009). Notably, symmetric dimethylation of H2A, H4R3 and H3R8 are considered general repressive marks and are usually targeted by PRMT5 in conjunction with chromatin-remodeler complexes involved in gene silencing, namely, human SWItch/Sucrose Non-Fermentable (hSWI/SNF) and SIN3 transcription regulator family member A/Histone deacetylase 2 (mSin3A/HDAC2) (Sharmistha Pal *et al.*, 2004; S. Pal *et al.*, 2003). On the

other hand, symmetric dimethylation of H3R2 (H3R2me2s) coincided with euchromatic regions which led to the recruitment of WDR5, a common component of co-activator complexes (Migliori *et al.*, 2012). Importantly, H3R2me2s was associated with transcriptional activation needed for cell-cycle withdrawal and differentiation in human cells (Migliori *et al.*, 2012). These findings indicate the indispensable histone-modifying role that PRMT5 plays in differentiation and embryonic developmental processes. PRMT5 has also been shown to regulate gene expression at the level of transcription elongation, translation and protein synthesis. For example, PRMT5-mediated methylation of a critical elongation factor, SPT5 was shown to regulate its interaction with RNA polymerase II, positively affecting its transcriptional elongation properties in response to viral and cellular factors (Kwak *et al.*, 2003). Additionally, PRMT5 promoted the recruitment of eIF4e to the 5′ cap of MYC, HIF1α and Cyclin D mRNAs, indicative of its role in cellular and molecular processes such as survival, proliferation, transcription elongation, cell cycle regulation and cellular adaptation (Lim *et al.*, 2014). Other less well-known but nevertheless essential roles for PRMT5 have been described, including regulation of ribosome biogenesis, metabolic gene reprogramming and Golgi apparatus integrity (L. Liu *et al.*, 2016; Ren *et al.*, 2010; Z. Zhou *et al.*, 2010).

Aside from histones, PRMT5 also dimethylates several key pleiotropic transcription factors (TFs), including p53, SREBP1, HOXA9, E2F-1, KLF4, PDCD4 and p65 (Shailesh *et al.*, 2018). Through modulation of these TFs, PRMT5 can enact a wider repertoire of transcriptional changes related to many essential cellular processes including cell cycle regulation, proliferation, hematopoiesis, DNA damage response and metabolic reprogramming, among others. For example, in endothelial cells, PRMT5 promotes transcription of pro-inflammatory and adhesion molecules by methylating HOXA9 which in turn promotes leukocyte adhesion and infiltration (Bandyopadhyay *et al.*, 2012). Similarly, a separate study showed that TNF-α-induced and PRMT5-mediated

symmetric dimethylation of R30 and R35 of p65 in endothelial cells activated transcription of chemokines CXCL10 and CXCL11, which are also involved in the recruitment of inflammatory cells (Harris *et al.*, 2014; Harris *et al.*, 2016). These examples serve to enhance our understanding of the role of PRMT5-mediated methylation of TFs and provide important mechanistic insight into how certain gene networks such as those pertaining to inflammation can be modulated by arginine methylation. In addition to p65 and HOXA9, other TFs mentioned above cooperate with PRMT5 to drive pro-inflammatory and tumorigenic processes. We will elaborate upon a few of these in the subsequent section.

PRMT5 also functions as a requisite factor in normal human hematopoiesis (F. Liu *et al.*, 2015). Lui *et al* showed that deletion of PRMT5 in hematopoietic cells resulted in dysfunction of hematopoietic stem and progenitor cells which ultimately led bone marrow failure (F. Liu *et al.*, 2015). Mechanistically, PRMT5-deficient hematopoietic stem cells exhibited severely attenuated cell surface expression of important cytokine receptors due to an impairment in the symmetric dimethylation of essential splicing proteins (Greenblatt *et al.*, 2016). This brings us to another well-characterized biological function of PRMT5: regulation of the spliceosome machinery. In eukaryotic cells, splicing is a critical process for increasing the diversity of the proteome and improper functioning of splicesomal proteins contributes significantly to the susceptibility or severity of several diseases. PRMT5 maintains splicing fidelity by mediating methylation of SmB, SmD1 and SmD3, facilitating their interaction with the survival motor neuron (SMN) complex and assembly into the pre-mRNA splicing core machinery (Bezzi *et al.*, 2013; Meister *et al.*, 2002). In the absence of PRMT5 however, defects in the splicing machinery produces unstable splice variants. One example is the generation of an abnormally spliced Mdm4 transcript in PRMT5-deficient hematopoietic cells. The mis-spliced Mdm4

product is unstable, leading to a reduction in Mdm4 protein levels and subsequent cell cycle arrest via aberrant activation of the p53 signaling pathway (Bezzi *et al.*, 2013; Litzler *et al.*, 2019). Clearly, PRMT5 functions as a master regulator of splicing in mammals which in turn serves to directly or indirectly affect many important downstream signaling pathways. The following section will provide a more detailed discourse on how these varied biological functions of PRMT5 are implicated in cancer.

1.3.2B Contributions of PRMT5 to cancer

Both the transcript and protein levels of PRMT5 are upregulated across several cancer types including but not limited to cancers of the bone marrow, breast, cervix, liver, pancreas, lung, ovaries, bladder, prostate and colon (Stopa *et al.*, 2015). Moreover, overexpression of PRMT5 is strongly correlated to advanced tumor stages and poor patient outcome (Shailesh *et al.*, 2018). Indeed, continuous efforts are underway to determine the clinical utility of PRMT5 overexpression as a possible diagnostic biomarker (Xiao *et al.*, 2019). Although reports of this nature are rapidly expanding, the mechanisms underlying PRMT5's suggested "oncogenic" role are yet to be fully elucidated. Thus far, PRMT5 has been implicated in the aberrant regulation of genes involved in cell proliferation, apoptosis, and DNA damage response (Xiao *et al.*, 2019). Accumulating evidence suggests that among these genes are several tumor suppressors which are epigenetically inactivated upon PRMT5-mediated methylation of histones. For instance, PRMT5-catalyzed H3R8 and H4R3 symmetric dimethylation has been shown to repress expression of tumor suppressor genes ST7 and NM23 which coincided with increased transformation of NIH3T3 fibroblast cells (Karkhanis *et al.*, 2011; Sharmistha Pal *et al.*, 2004). Likewise, in leukemia and lymphoma cells, upregulation of human SWI/SNF-associated PRMT5 was shown to be involved in the

transcriptional repression of retinoblastoma (RB) family of tumor suppressors including RB1, RBL1 and RBL2 (Wang *et al.*, 2008). In the same study, depletion of PRMT5 expression in B-cell chronic lymphocytic leukemia cells, WaC3CD5, abolished methylation of H3R8 and H4R3 which restored RB gene expression and hamper cancer cell proliferation (L. Wang *et al.*, 2008). In another study, upregulation of PRMT5 in EBV-driven transformed B-lymphocytes promoted recruitment of an HDAC3-repressive complex to the promoter of another tumor suppressor, Protein Tyrosine Phosphatase Receptor-type O (PTPROt) (Alinari *et al.*, 2015). Expectedly, re-expression of PTPROt following PRMT5 inhibition led to mitigation of the malignant phenotype, suggesting PRMT5 inhibition could serve as a promising strategy for treating B-cell lymphomas. In a more recent study, overexpression of PRMT5 was detected in a large cohort of human gastric tumors where depletion of PRMT5 resulted in reduced cell growth and metastasis. Moreover, its upregulation contributed to increased recruitment of DNA methyltransferase 3A (DNMT3A) to the promoter region of the tumor suppressor gene, Iroquois homeobox 1 (IRX1), thus coupling histone and DNA methylation to promote gene silencing (X. Liu *et al.*, 2018). Collectively, these results highlight the crucial role that PRMT5 overexpression plays in epigenetically repressing the transcription of key tumor suppressor genes and suggest a causal role of the associated elevated methylation of histones in promoting tumorigenesis.

Aside from histone-related epigenetic modulation, evidence supporting PRMT5-mediated alteration of tumor suppressor function is seen through its direct methylation of p53 in osteosarcoma cells. Dubbed the “guardian of the genome”, p53 is activated in response to DNA damage which results in apoptosis or cell-cycle arrest. Following DNA damage, PRMT5 was shown to methylate p53 within its oligomerization domain to modify its DNA binding activity and thus trigger altered target gene specificity (Jansson *et al.*, 2008). For instance, ectopic PRMT5 enhanced the

expression of a p53-induced cell-cycle arrest gene program while reducing expression of signature genes related to apoptosis. Accordingly, PRMT5 depletion prompted apoptosis, suggesting that PRMT5 influences the outcome of the DNA damage response (Jansson *et al.*, 2008). This differential outcome is significant in the context of anti-cancer therapy as p53-mediated apoptosis is considered a desirable outcome of DNA-damaging drugs. However, during p53-induced cell-cycle arrest, the cell attempts to repair DNA damage, perhaps with the assistance of genes such as Gadd45 that enhance its repair capacity (Pucci *et al.*, 2000). Consequently, PRMT5-catalyzed and p53-mediated induction of cell-cycle arrest not only favors survival and cell cycle arrest over apoptosis but may also interfere with and reduce the efficacy of drugs that target mitosis.

In addition to its gene repressive functions and modulation of tumor repressors, PRMT5 overexpression is also intrinsically linked to enhanced gene activation which has been shown to promote cancer malignancy by orchestrating a cascade of downstream oncogenic signaling events. One pertinent example is that of the role of PRMT5 in promoting prostate cancer cell growth through epigenetic transcriptional activation of the androgen receptor (AR) (Deng *et al.*, 2017). This receptor plays pivotal roles in prostate cancer and remains the major target of androgen blockade drugs used to treat castration-resistant prostate cancer (CRPC) (Rathkopf *et al.*, 2013). However, quite often, prostate cancer cells adapt to these therapies and resistance emerges due to upregulation of AR expression, underscoring the need to identify mechanisms that lead to altered AR levels. In a recent study, PRMT5 and AR expression were concurrently elevated in prostate cancer tissues compared to their benign prostatic counterparts. Mechanistically, PRMT5 was recruited to the AR promoter through its interaction with Sp1, a critical transcriptional factor responsible for AR transcription. The net outcome of PRMT5 overexpression was upregulated AR

expression, facilitated by enriched symmetric dimethylation of H4R3 near the proximal AR promoter. Interestingly, this signifies a previously unreported activating role for the otherwise-known repressive H4R3me2s mark. Furthermore, knockdown or pharmacologic inhibition of PRMT5 suppressed the growth of AR-positive but not AR-negative prostate cancer cells (Deng *et al.*, 2017). These findings argue for the development of new and compelling personalized targeted therapies based on exploiting certain vulnerabilities conferred by PRMT5-mediated activation of genes such as AR.

Further evidence in support of the gene activating role of PRMT5 in terms of its ability to enhance the proliferative capabilities of cancer cells comes from studies in CRC cells, which is the cancer of primary interest in the current dissertation work. In CRC cells, PRMT5-induced H3R8 and H4R3 symmetric methylation correlated with increased transcription of the Fibroblast-derived Growth Factor Receptor-3 (FGFR-3) and eukaryotic elongation Initiation Factor-4E (eIF4E) genes (B. Zhang *et al.*, 2015). Conversely, inhibition of PRMT5 in response to AMI-1 treatment reduced the expression of these genes while inducing apoptosis in CRC cells and in a mouse CRC xenograft model (B. Zhang *et al.*, 2015). In a separate study, overexpression and hyperactivity of PRMT5 in a subgroup of CRC cells was associated with poor clinical outcome via direct methylation and subsequent inhibition of E2F-mediated growth control, thereby contributing to uncontrolled cell proliferation (Cho *et al.*, 2012). Intriguingly, a recent study by Zheng *et al* (2017) demonstrated a novel functional link between PRMT5 and ulcerative colitis (UC), a major risk factor for CRC initiation. Depletion or pharmacological inhibition of PRMT5 enhanced the levels and function of regulatory T cells (Tregs) while attenuating UC in a mouse model of dextran sulfate sodium (DSS)-induced colitis. Furthermore, blockade of PRMT5 in Tregs also led to a decreased transcription of pro-inflammatory cytokines TNFα, IL6, and IL-13, implicating a role for

PRMT5-mediated gene regulation in the modulation of inflammatory responses (Y. Zheng *et al.*, 2017). Remarkably, our lab previously demonstrated a role for PRMT5 in pro-inflammatory cytokine-activated transduction pathways. We found that NF-κB is dimethylated on R30 in response to IL-1β-stimulation of PRMT5, which was found to profoundly regulate NF-κB activation of more than 75% of its target genes. These genes include TNF-α, Interleukin 8 (IL8), and mitogen-activated protein kinase kinase kinase 8 (MAP3K8), all implicated as being essential in inflammation and cancer (Wei *et al.*, 2013). Similarly, later studies from another group showed that PRMT5-mediated p65 methylation was essential for NF-κB-dependent induction of CXCL10 and CXCL11, chemokines critical for the recruitment of immune cells to inflammatory sites (Harris *et al.*, 2014; Harris *et al.*, 2016). Thus, targeting PRMT5 presents a promising therapeutic approach to moderating chronic inflammatory pathologies, including cancer.

Collectively, the spectrum of PRMT5 target proteins (**Table 1**) provides strong evidence for its complicated and sophisticated involvement in a wide array of signaling pathways which also implies that PRMT5 might function in the context of multiple oncogenic drivers (**Figure 7**). In the future, it will be interesting to explore the possible mutual interplay between these signaling pathways and varied substrates of PRMT5. Importantly, cataloging these nodes of crosstalk will provide crucial insight for the rational development of anti-cancer combination therapies aimed at targeting multiple signaling dependencies at once.

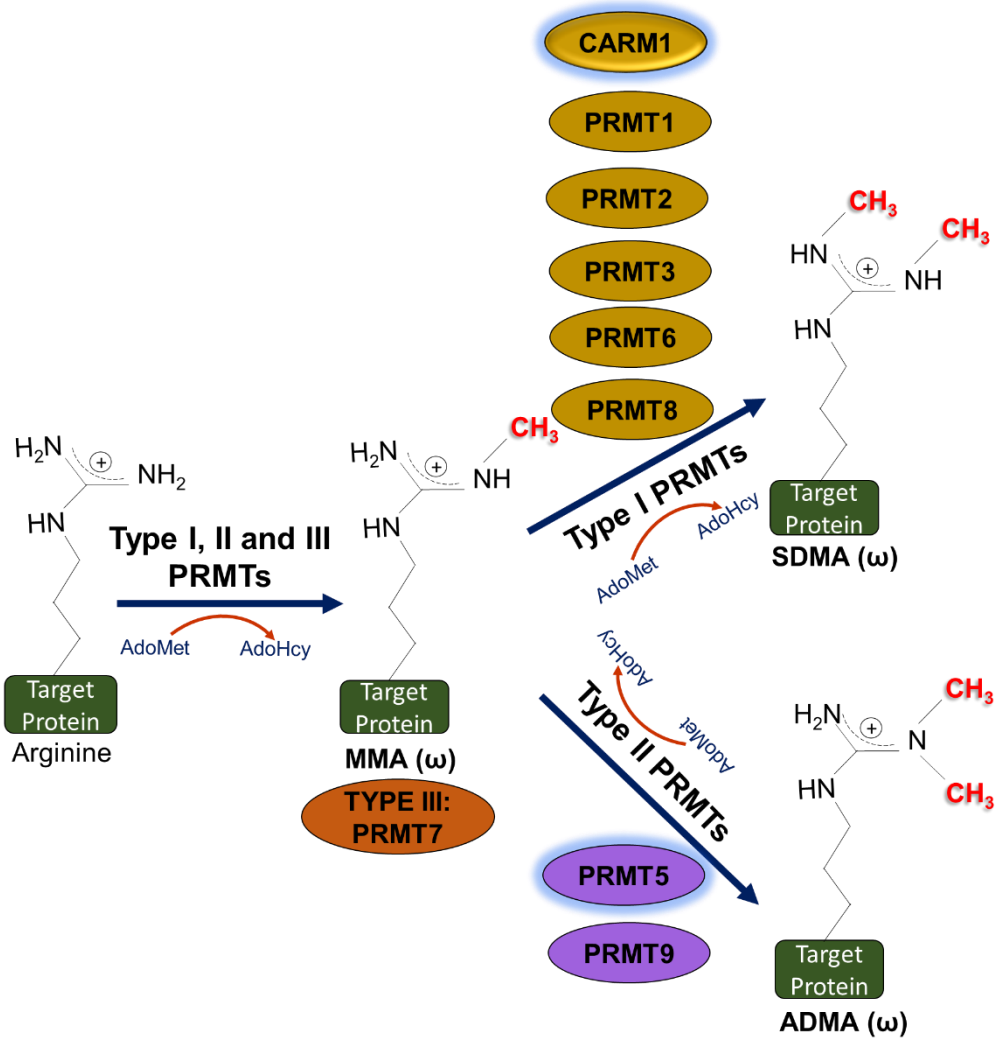


Figure 5: Classification of PRMT family members based on methylation patterns (Bedford *et al.*, 2009)**.** Three distinct types of methylated arginine residues exist in mammalian cells: monomethylarginine (MMA), asymmetric dimethylargine (ADMA) and symmetric dimethylarginine (SDMA). All PRMT family members catalyze the formation of MMA as an intermediate by adding one methyl group on of the terminal (**ω**) guanidine nitrogen atoms. ADMA marks are catalyzed by Type I enzymes of which CARM1 is the most prominent. ADMA marks are generated by the addition of two methyl groups on the same terminal (**ω**) guanidine nitrogen atom whereas SDMA marks are formed by the addition of a methyl group on each of the two terminal nitrogens. This is catalyzed by Type II PRMTs of which PRMT5 is the most prominent. PRMT7 is the only type III enzyme, which mainly generates MMA. The main enzymes for each type of methylation pattern are indicated.

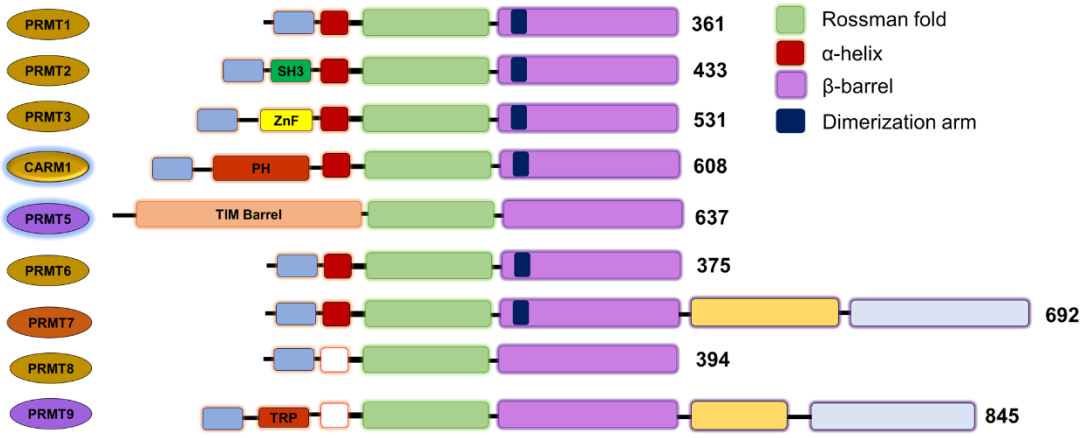


Figure 6: Domain architecture of human PRMTs (Schapira *et al.*, 2014)**.** There are currently nine members of the mammalian PRMT family, which harbor highly conserved domains including the catalytic domain (Rossman fold), α-helix and β-barrel. The number of residues is indicated at the C terminus of the PRMTs and additional signature domains are indicated. SH3: SRC Homology 3 Domain; ZnF: Zinc finger; PH: Pleckstrin homology; TIM: triosephosphateisomerase; TRP: tetratricopeptide.

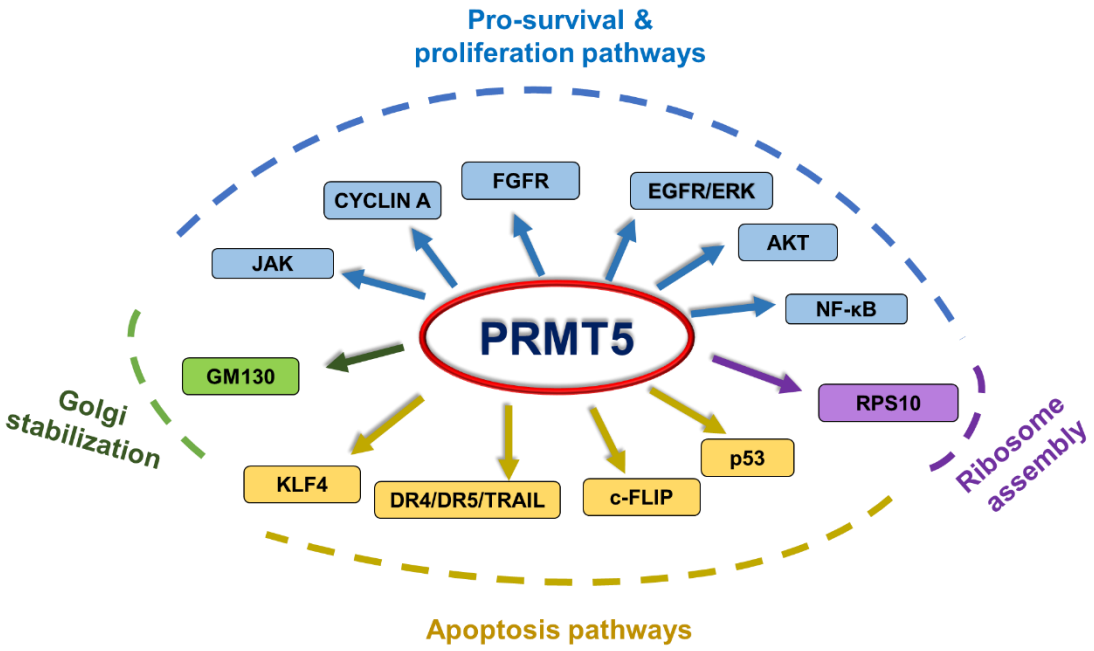


Figure 7: Schematic model of various PRMT5-regulated signaling molecules, pathways and cellular processes (Karkhanis *et al.*, 2011)**.** PRMT5 regulates various cellular processes via different mechanisms including modification of key regulatory factors such as transcription factors (e.g., p53, KLF4, NF-κB), receptors (e.g., EGFR, FGFR3), Golgi proteins (GM130) and ribosomal proteins (RPS10). Together, these PRMT5-mediated alterations profoundly affect signaling pathways related to apoptosis, proliferation, pro-survival, ribosomal assembly and Golgi stabilization.

1.3.3 Regulation of PRMT5 by interacting partners

PRMT5 can be regulated at multiple levels (**Figure 8**) and is principally active as part of a larger multimeric complex which associates with a broad range of interacting partners (Antonysamy, 2017). By and large, these interactions regulate its activity and/or substrate specificity. MEP50 is considered to be a major cofactor of PRMT5 and together, they form a catalytically active hetero-octameric complex *in vitro* which also serves to define its substrate specificity as well as its distributive catalytic mode of action (Antonysamy, 2017). However, whether MEP50 is obligatory for PRMT5 function *in vivo* remains uncertain. Nevertheless, the MEP50-PRMT5 interaction appears to primarily promote methylation of histones whereas other cofactors such as Blimp1, Menin/Men1 and members of the hSWI/SNF chromatin remodeling complex may elicit methylation specificity towards other substrates including nucleosomes (Stopa *et al.*, 2015). Another cofactor, cooperator of PRMT5 (COPR5), was found to predominantly interact with PRMT5 in the nucleus and appeared to be partly responsible for PRMT5's transcriptional repressor activities (Lacroix *et al.*, 2008). Interestingly, when complexed with COPR5, PRMT5 also preferentially methylated H4R3 over H3R8, introducing yet another layer of complexity that involves more subtle distinguishing between histone substrates (Karkhanis *et al.*, 2011). Likewise, other proteins of the PRMT5 interactome have been shown to trigger preferential recruitment of PRMT5 for the repression of gene expression. Association of bromodomain protein 7 (BRD7) with the PRMT5-containing hSWI/SNF chromatin remodeling complex correlated with PRMT5 recruitment to, and subsequent hypermethylation of H3R8 and H4R3 at the promoters of ST7 and RBL2 (Tae *et al.*, 2011).

Interestingly, the interaction of one binding partner of PRMT5 can also preclude its complex with another, which may further dictate substrate selectivity. The mutually exclusive interaction of PRMT5 with the adaptor proteins, plCln and RioK1 is a chief

example. Association of RioK1 with PRMT5 targets PRMT5-mediated methylation of nucleolin, a phosphoprotein involved in the synthesis and maturation of ribosomes. On the other hand, pICln precludes the binding of PRMT5 to Riok1 and instead, directs recruitment of Sm proteins for their symmetric dimethylation by PRMT5 to form small nuclear ribonucleoproteins (Guderian *et al.*, 2011). Furthermore, PRMT5-pICln was also shown to inhibit the methylation of histones. In total, these findings provide key mechanistic insight into how protein-protein interactions (PPIs) orchestrate the distinction between different substrate proteins, which may be useful for the rational design of PRMT5-specific inhibitors that perturb these PPIs.

1.3.4 Regulation of PRMT5 by existing substrate modifications

The existence of other types of PTMs can also alter the activity of PRMT5 towards a given substrate. For instance, pre-existing acetyl marks on H4K5 and H4K16 were shown to facilitate deposition of PRMT5-mediated H4R3me2s marks. However, whether the reverse is true where H4R3me2s promotes these acetylation events remains to be established (Feng *et al.*, 2011). Conversely, phosphorylation of H2AS1 and H4S1 had an inhibitory effect on PRMT5 activity (Ho *et al.*, 2013). Stopa *et al* hypothesized that the latter may be due to steric hindrance conferred by the bulkier phosphorylation moiety (Stopa *et al.*, 2015). Together, these reports not only highlight another mechanism by which arginine methylation is regulated, via induction of crosstalk between different PTMs, but signifies the complex interplay between PRMTs and other classes of enzymes including histone acetyltransferases (HATs) and kinases.

1.3.5 Regulation of the subcellular localization of PRMT5

Although predominantly cytoplasmic, PRMT5 has been shown to localize to the nucleus, Golgi apparatus and cell membrane. Intriguingly, the subcellular localization of

PRMT5 appears to be temporally and dynamically governed by factors such as developmental stage, cell-type, and disease state. For example, during early embryonic stages PRMT5 interacts with Blimp1 and is localized to the nucleus in mouse primordial germ cells (Ancelin *et al.*, 2006). This is linked to high levels of symmetric dimethylation of H2A and H4R3. However, by E11.5 Blimp1 is downregulated and PRMT5 persists in the cytoplasm, thus decreasing nuclear H2A/H4R3 methylation levels. This shuttling of PRMT5 between the nucleus and the cytoplasm ensures temporal silencing of transposons and maintenance of genomic integrity during extensive, global DNA demethylation (Ancelin *et al.*, 2006). Conversely, in human zygotes and early embryos, PRMT5 is cytoplasmic, but thereafter becomes nuclear up until the early E3.5 blastocyst stage which coincides with enriched H2A/H4R3me2s levels. It then becomes mostly cytoplasmic once again (Kim *et al.*, 2014). In embryonic stem cells, PRMT5 is also co-upregulated with STAT3 in the cytoplasm to maintain pluripotency via repression of differentiation genes (Tee *et al.*, 2010).

Compartmentalization of PRMT5 not only varies across different cell types and developmental stages but is also implicated as holding prognostic value in various cancers. In fetal germ cells, PRMT5 is cytoplasmic; however, in Leydig and adult testicular cells, it is nuclear. This pattern is reversed in Leydig tumor cells, in which PRMT5 shows an increased cytoplasmic localization (Eckert *et al.*, 2008). Moreover, cytoplasmic localization of PRMT5 was shown to correlate with poor prognosis in high-grade, poorly differentiated lung adenocarcinomas, whereas a predominantly nuclear PRMT5 was identified in low-grade tumors (Ibrahim *et al.*, 2014; Shilo *et al.*, 2013). In prostate cancer cells, PRMT5 was found to function in opposite ways in the cytoplasm and nucleus. While nuclear PRMT5 was observed in benign prostate epithelium, it primarily localized to the cytoplasm in neoplastic and malignant prostate cancer tissues (Gu, Li*, et al.*, 2012). Correspondingly, cytoplasmic PRMT5 promoted cell proliferation in

a methyltransferase-dependent manner whereas nuclear localization of PRMT5 had a growth-inhibitory effect (Gu, Li*, et al.*, 2012). Additional studies showed that compared to their benign counterparts, melanoma tissues exhibited higher cytoplasmic PRMT5 (Nicholas *et al.*, 2013). By contrast, nuclear PRMT5 was associated with poor overall survival in oropharyngeal squamous cell carcinomas (Kumar *et al.*, 2017). Overall, the compartment-related functional dichotomy described above appears to be linked to the association of nuclear or cytoplasmic PRMT5 with, and subsequent silencing and/or activation of various molecular targets, albeit the exact mechanisms are yet to be determined.

PRMT5 was shown to also localize to the Golgi and to be in proximity of the cell membrane. In the Golgi, it associates with and methylates GM130, a Golgi matrix protein required for the transitioning of ER vesicular to Golgi-specific cisternal membranes. Depletion of PRMT5 led to impaired Golgi ribbon formation indicating the critical role of PRMT5 in maintaining the overall integrity of this organelle (Z. Zhou *et al.*, 2010). At the cell membrane, PRMT5 has been shown to methylate R1175 on EGFR, dampening the effect of EGF-stimulated ERK activation (Hsu *et al.*, 2011).

Taken together, these findings clearly suggest that symmetric arginine methylation is a tightly controlled PTM as evidenced by a strong correlation between PRMT5 localization and the distinct functional outcomes observed. Mechanistically, the cytoplasmic localization may be explained by deletion analyses which revealed that PRMT5 harbors three nuclear exclusion signals (NESs) (Gu, Gao*, et al.*, 2012). However, since other localization motifs (e.g., NLS) are yet to be identified within the PRMT5 protein, future studies to determine specific signaling cues or binding partners that initiate its shuttling to other organelles is imperative.

1.3.6 Transcriptional and post-transcriptional regulation of PRMT5

It is widely appreciated that the overexpression of PRMT5 contributes to the development and progression of several human cancers. However, the mechanisms underlying transcriptional regulation of PRMT5 expression remain elusive. Recent studies identified nuclear transcription factor Y A (NF-YA) as a critical transcription factor that binds CCAAT boxes within the promoter region of PRMT5. Knockdown of NF-YA resulted in loss of PRMT5 expression and consequent growth defects of prostate cancer cells in a PKC/c-Fos-dependent manner (H.-T. Zhang *et al.*, 2014). Another pertinent study using a mouse lymphomagenesis model, showed that oncogenic MYC could directly upregulate PRMT5 whereas in AML cells, the polymerase-associated factor complex (PAFc) indirectly amplified its expression (Koh *et al.*, 2015; Serio *et al.*, 2018). Interestingly, a more recent study provided evidence for the possible epigenetic regulation of PRMT5 expression. Depletion of the HAT, N-alpha-acetyltransferase 40 (NAA40) in CRC cells was shown to significantly reduce and enhance the enrichment of activating N-acH4 and repressive H3K27me3 marks, respectively, at the promoter of PRMT5. This corresponded to decreased levels of H4R3me2 through repression of PRMT5 expression. The net outcome was altered expression of key oncogenes and tumor suppressor genes regulated by PRMT5 which ultimately led to inhibition of CRC cell growth (Demetriadou *et al.*, 2019). Altogether, these studies reveal another complex node in the multi-layered regulation of PRMT5. However, the paucity of reports in this area suggests an overall lag in uncovering the most critical transcription factors that drive PRMT5 expression, yet this knowledge is essential to effectively exploiting these factors for novel anti-cancer therapies.

Lately, several correlative studies suggest that PRMT5 is also regulated at the post-transcriptional level by the 3^{l}-UTR targeted action of several miRNAs including miR-92b and miR-96. In a panel of mantle cell lymphoma (MCL) and lymphoid cancer cell

lines, decreased miR-92b and miR-96 expression was shown to augment PRMT5 translation which was accompanied by increased H3R8/H4R3 methylation and subsequent repression of ST7. Furthermore, re-expression of miR-92b and miR-96 miRNAs resulted in a corresponding decrease in PRMT5 protein levels (Sharmistha Pal *et al.*, 2007). Similar effects were observed in transformed B cell chronic lymphocytic leukemia (B-CLL) cells. Intriguingly, recent studies also suggest that PRMT5 participates in several regulatory feedback circuits with miRNAs in which PRMT5 might promote its own expression through epigenetic silencing of specific miRNA gene programs, suggesting a mutual interplay and coordinated regulation between PRMT5 and miRNAs for the maintenance of cancerous phenotypes (Vrajesh Karkhanis *et al.*, 2014). Arguably, whether targeting these upstream determinants of PRMT5 expression will likely lead to a general clinical advantage for patients, remains to be elucidated. Hence, future translational studies are needed to identify patient populations that could benefit from multipronged targeting of PRMT5, involving direct inhibition PRMT5 activity as well blocking upstream factors that contribute to its aberrant expression.

1.3.7 Regulation of PRMT5 by posttranslational modifications

Like other methyltransferases, PRMT5's activity is also fine-tuned by PTMs such as phosphorylation, methylation and ubiquitination. Tyrosine phosphorylation constitutes one of the earliest evidences of posttranslational regulation of PRMT5. Phosphorylation of Tyr304 and Tyr307 by oncogenic $JAK2^{V617F}$ mutant was shown to downregulate the methyltransferase activity of PRMT5 via disruption of the PRMT5-MEP50 interaction to promote myeloproliferation (F. Liu *et al.*, 2011). In another study, phosphorylation of a threonine residue, T634, functioned to target PRMT5 to the plasma membrane via modulation of a PRMT5 interaction switch involving PDZ and 14-3-3 (Espejo *et al.*, 2017). In the current study, we reveal a novel regulatory role for serine phosphorylation

of PRMT5 within the TIM-Barrel domain of PRMT5 in CRC cells. In the subsequent sections, we demonstrate that IL-1β-inducible phosphorylation of S15 positively regulates its methyltransferase activity and complexing with the p65 subunit of NF-κB which may undergird one aspect of its tumor-promoting role in CRC cells. Interestingly, another recent study by Lattouf *et al* also identified threonine phosphorylation sites within the same TIM-Barrel motif of PRMT5 in breast cancer cells. Point mutations of T132, T139 and T144 residues on PRMT5 resulted in its dissociation from its binding partners MEP50, pICln and RioK1, leading to overall lower methyltransferase activity (Lattouf *et al.*, 2019). These latter studies argue for a potentially new regulatory mechanism involving phosphorylation of residues within the TIM-Barrel, a domain unique to PRMT5. Furthermore, identification of these PTMs across different cancer tissues could potentially have clinical implications in identifying specific subsets of patients where phosphorylation of PRMT5 may mediate its tumor-associated roles.

Apart from phosphorylation, the role of other PTMs in regulating PRMT5 has been described. For instance, Nie *et al* identified methylation of PRMT5 as a positive regulator of its catalytic activity. CARM1 was recently shown to directly interact with, and asymmetrically dimethylate PRMT5 on R505, to promote its oligomerization and thus enhance its methyltransferase activity (Nie *et al.*, 2018). On the other hand, polyubiquitination of PRMT5 by the ligase CHIP was found to be essential to the negative regulation of PRMT5 expression via K48-linked ubiquitin-dependent proteasomal degradation in pancreatic cancer cells (H. T. Zhang *et al.*, 2016). Taken together, these findings support a complex model of the regulation of PRMT5 by PTMs that define its methyltransferase activity, protein-protein interactions and stability. To our knowledge, the work outlined in this thesis is the first report of a cytokine-induced modification on PRMT5. This is a significant observation since cytokines play critical roles in modulating tumor-related processes. Indeed, further understanding of the

extracellular clues that underlie the other afore-mentioned PTMs may serve as the basis for designing novel therapeutic approaches for PRMT5-driven diseases, including cancer.

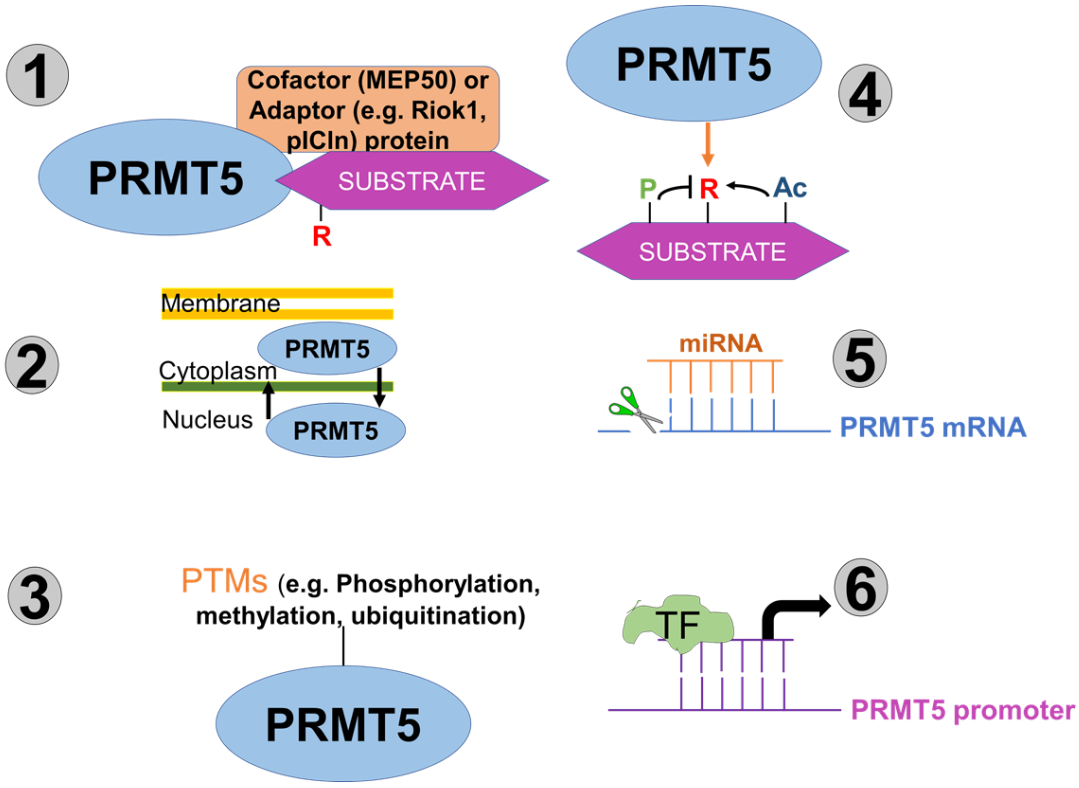


Figure 8: Multi-level regulation of PRMT5. **1)** Cofactor (e.g., MEP50) and adaptor proteins (e.g., RioK1) bridge the interaction between PRMT5 and specific substrates, targeting them for methylation. **2)** PRMT5 can be regulated by shuttling between the nucleus and the cytoplasm, thus positioning it to interact with and methylate different substrate proteins. **3)** Distinct PTMs such as phosphorylation, methylation and ubiquitination have been shown to modulate the activity of PRMT5.**4)** The pre-existence of other PTMs on the substrate can facilitate (e.g., acetylation) or prevent (e.g., phosphorylation) adjacent arginine methylation. **5)** PRMT5 can be targeted for mRNA cleavage and translational repression through the action of miRNAs. Finally, **6)** The transcriptional levels of PRMT5 can also be modulated upstream by transcription factor-binding to its promoter region.

Table 1: Effect of PRMT5-mediated methylation of different substrates

	KNOWN PRMT5 SUBSTRATE	REPORTED SITE/MOTIF	FUNCTION	REFERENCE
HISTONES	H2A	R3	Transcriptional activation or repression	Tee et al., 2010
	H3	R2; R8	Transcriptional activation; repression	Yuan et al., 2012; Tsai et al., 2013; Pal et al., 2004 & 2007
	H4	R3	Transcriptional repression	Yue et al., 2013; Girardot et al., 2014
NON-HISTONE PROTEINS	ASH2L	R296	Unclear	Butler et al., 2011
	CBP-1	R234	Negatively regulates DNA-damage-induced apoptosis in *C. elegans*	Yang et al., 2009
	CF I(m)68	GAR motif	Regulates processing of 3^{i} ends of precursor mRNAs	Martin et al., 2010
	EBNA-1	GAR motif	Alteration of EBNA-1 localization	Shire et al., 2006
	EGFR	R1175	Attenuates EGFR-mediated ERK activation	Hsu et al., 2011
	FEN1	R192	Regulates interaction with PCNA and localization of FEN1 to DNA replication/repair foci	Guo et al., 2010
	Eno-1	R50	Enhances Eno-1 cell surface levels and invasiveness of lung cancer cells	Zakrzewicz et al., 2018
	FCP1	R913, R916	Promotes methylation of free H4	Amente et al., 2005
	HOXA9	R140	Regulates leukocyte adhesion molecules in endothelial cells	Bandyopadhyay et al., 2012
	LSm4	Unknown	Spliceosome assembly	Brahms et al., 2001
	MBP	R107	Regulates myelination	Baldwin and Carnegie, 1971
	MBD2	RG motif	Inhibit formation of transcriptional repression complex	Tan and Nakielny, 2006
	NaV1.5	Unknown	Increases NavV1.5 cell surface expression and current density	Beltran-Alvarez et al., 2013

Table 1 (continued): Effect of PRMT5-mediated methylation of different substrates

KNOWN PRMT5 SUBSTRATE	REPORTED SITE/MOTIF	FUNCTION	REFERENCE
Nucleolin	RG motif	Required for interaction with RNA	Guderian et al., 2011
Nucleoplasmin	GAR motif	Regulates pluripotency	Wilczek et al., 2011
p53	R333; 335; 337	Regulates p53 target gene specificity	Jansson et al., 2008
p65	R30; R35; R174	Activates p65 transactivation, DNA–binding and target gene expression; promotes CXCL10 and CXCL11 induction	Wei et al., 2014; Harris et al., 2014 & 2016
PDCD4	R110	Enhance breast tumor growth	Powers et al., 2011
RNAPII	R1810	Required for recruitment of SMN	Zhao et al., 2016
RPS10	R158, 160	Promotes proper assembly of ribosomes, protein synthesis and optimal cell proliferation	Ren et al., 2010
RUVBL1	R205	Regulates double-strand break repair	Clarke et al., 2017
SKI	R8	Promotes melanomagenesis	Tamiya et. al, 2018
SmD1	GAR motif	Spliceosome assembly	Brahms et al. 2001
SmD3	GAR motif	Spliceosome assembly	Brahms et al. 2001
SPT5	R698	Stimulate transcription elongation	
ZNF326	R175	Promotes proper splicing; involved in tumor cell proliferation and migration	Rengasamy et. al, 2017

1.4 Summary and Hypotheses

A plethora of findings suggest that the p65 subunit of NF-κB undergoes a variety of PTMs and that these events are critical to modulating the transcriptional output NF-κB (Huang *et al.*, 2010). Importantly, recent findings from our lab represent the first report of PRMT5-mediated arginine methylation of p65 at R30 as being another critical modification involved in regulating a subset of IL-1β-inducible NF-κB-dependent genes (Wei *et al.*, 2013). Moreover, in the same study, supporting evidence from illumina array data suggests that majority of the genes (~85%) downregulated by the R30A mutant were also downregulated by depletion of PRMT5. The results from this cross-comparison are significant since PRMT5 is highly overexpressed across many cancer types in which constitutive activation of NF-κB is also implicated, including CRC. As such, we hypothesized that high levels of this enzyme may promote CRC malignancy at least in part, by facilitating aberrant NF-κB-induced gene expression. Indeed, findings from the current and previous work reveal that overexpression of PRMT5 significantly augments NF-κB activation and target gene transcription concurrent with promoting the migration, proliferation and anchorage-independent growth of CRC cells. However, the precise mechanisms governing this PRMT5/NF-κB regulatory axis are unknown. In this regard, the work described here focuses on the phospho-mediated regulation of this axis based on our discovery of a novel S15 phosphorylation site on PRMT5. We propose that phosphorylation of PRMT5 at S15 is critical to its activation of NF-κB and partially contributes to the tumor-associated roles of PRMT5 in CRC. In summary, the findings from this study highlight a new regulatory mechanism of PRMT5 via IL-1β-inducible serine phosphorylation that could have clinical implications for treating CRC and other cancers.

CHAPTER 2: METHODS

2.1 *In Vitro* experiments

2.1.1 Liquid chromatography-tandem mass spectrometric analysis

Coomassie-stained SDS-PAGE gel band containing Flag-PRMT5 protein was subjected to in-gel tryptic digestion. Flag-PRMT5 gel pieces were subjected to destaining and reduction of cysteine residues using 50% acetonitrile in 100 mM ammonium bicarbonate and 100% acetonitrile followed by treatment, followed by 20 mM DTT at room temperature for 60 min. Alkylation with 55 mM iodoacetamide for 30 min was performed in the dark. The solution was removed and the gel pieces were washed with 100 mM ammonium bicarbonate and dehydrated in acetonitrile. Gel pieces were then dried in a SpeedVac centrifuge, and proteolytically digested by rehydration overnight at 37 °C in 50 mM ammonium bicarbonate containing sequencing grade modified trypsin (Promega, WI). Extracted peptides were treated with 50% acetonitrile in 5% formic acid, dried and reconstituted in 0.1% formic acid for mass spectrometry analysis. Analysis of proteolytic digests was performed by using an LTQ Orbitrap XL linear ion-trap mass spectrometer (Thermo Fisher Scientific), coupled with an Ultimate 3000 HPLC system (Dionex). The digests were injected onto a reverse-phase C18 column (0.075 × 150 mm, Dionex) equilibrated with 0.1% formic acid/4% acetonitrile (vol/vol). A linear gradient of acetonitrile from 4 to 40% in water in the presence of 0.1% formic acid over a period of 45 min was used at a flow rate of 300nL/min. The spectra were acquired by data dependent methods, consisting of a full scan (m/z 400–2,000) and then tandems on the five most abundant precursor ions. The previously selected precursor ions were scanned once during 30 s and then were excluded for 30 s. The obtained data were analyzed by Mascot software (Matrix Science) against customized PRMT5 protein database with the setting of 10 ppm for precursor ions and 0.8 Da for product ions. Carbamidomethylation of cysteine was set as fixed modification, while

oxidation of methionine, phosphorylation of serine, threonine, and tyrosine were set as variable modifications. The tandem mass spectra of candidate-modified peptides were further interpreted manually.

2.1.2 Cell lines & materials

The 293IL1R (denoted as HEK293) cell line was previously described by Lu *et al,* 2010 (Lu *et al.*, 2010). HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) (GE Healthcare), supplemented with 1% of penicillin/streptomycin and 5% fetal bovine serum (FBS). CRC cell lines HT29, DLD1 and HCT116 were procured from American Type Culture Collection (ATCC) (Manassas, VA) and were cultivated in RPMI (Roswell Park Memorial Institute, Buffalo, NY) 1640 medium supplemented with 100 units/ml penicillin, 100 g/ml streptomycin, and 5% fetal bovine serum (FBS). Cell lines were authenticated using 9 Marker STR Profile by IDEXX Bioresearch.

2.1.3 Generation of stable PRMT5-overexpressing cell lines

Flag-tagged WT-PRMT5 cDNA construct was amplified from total mRNA derived from HEK293 cells and cloned into the pLVX-IRES-puro vector (Lu *et al.*, 2010). The Flag-S15A-PRMT5 and E444D mutants of PRMT5 was generated using the QuikChange II XL Site-Directed Mutagenesis Kit from Agilent Technologies. Primers were designed using the Agilent Technologies QuikChange Primer Design online software and are outlined in **Appendix C**. Constructs were transfected into HEK293C6 and CRC cell lines as essentially described by Lu *et al* using Lipofectamine and PLUS reagents (Life Technologies/Invitrogen, Carlsbad, CA) (Lu *et al.*, 2010). For the generation of stable PKCι knockdown cell lines, a pool of 5 shRNAs constructs against PKCι were employed (Sigma-Aldrich). The respective lentiviral plasmids containing empty vector, Flag-WT-PRMT5, Flag-S15A-PRMT5 or shRNA against PKCι were

transected into a high efficiency 293T packaging cell line to produce high-titer viral preps that were used to infect HEK293 or a panel of CRC cells. Upon 48h of infection, cells were selected under 1μg/mL of puromycin and verified for the expression of various proteins by western blot analyses.

2.1.4 Western blotting and antibodies

Cells were cultured to about 90-95% confluence. Whole cell samples were collected in 1X phosphate buffered saline (PBS), centrifuged at 5,500 rpm for 5 min and lysed using Radio Immunoprecipitation Assay buffer (RIPA buffer: 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 8.0, 1 mM PMSF and protease inhibitors). The protein concentration of each sample was tested using the Protein Assay Reagent (Biorad) assay and absorbance values measured using a Genesys 10S Vis spectrophotometer (Thermo Fisher Scientific). Equal protein concentrations in 2X SDS sample loading buffer [100mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200mM β-mercaptoethanol] were loaded and separated by SDS/PAGE gels followed by further assessment by western blotting. Different antibodies were used to detect the target proteins of interest, obtained from the following commercial sources: anti-PRMT5 (Abcam, ab109451), anti-Flag (Sigma-Aldrich, F1804), and anti-p65 (Santa Cruz Biotechnology, sc-109); anti-PKCι (Proteintech, 66493-1-Ig). For cell fractionation experiments, cytoplasmic and nuclear fractions were subject to SDS-PAGE and probed with anti-LaminB1 (Proteintech, 12987-1-AP); anti-α-tubulin (Cell Signal, 2144S). The enhanced chemiluminescence (ECL) detection method (PerkinElmer) was used to detect protein signals.

2.1.5 NF-κB luciferase assay

NF-κB luciferase assays were conducted by infecting respective stable cell lines with a lentivirus originally generated in 293T cells using the κB-luciferase construct p5XIP10 κB and Lipofectamine™ LTX Reagent and PLUS Reagents (Thermo Fisher Scientific). Luciferase activity was assayed 48 h later using the Luciferase Assay System with Reporter Lysis Buffer kit (Promega, Fitchburg, WI). The κB-luciferase plasmid p5XIP10 κB contains five tandem copies of the NF-κB DNA binding site derived from the IP10 gene (an established target gene of NF-κB) upstream of a luciferase reporter gene. Luciferase activity was quantified using a Synergy H1 Multi-Mode Reader (BioTek Instruments Inc., Winooski, VT). All readings were normalized to total protein amount per condition.

2.1.6 Illumina microarray & quantitative PCR

Microarray and qPCR experiments were carried out as essentially described (Wei *et al.*, 2013). Briefly, control or HEK293 cells with WT-PRMT5 or S15A-PRMT5 overexpression were cultured to ~90% confluence and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Total isolated RNA was used to prepare cDNA using the SuperScript III First-Strand Synthesis PCR System (Invitrogen, Carlsbad, CA). cDNA was labeled with biotin-UTP using the Illumina Total Prep RNA amplification kit (Ambion/Applied Biosystems, Foster City, CA), hybridized to Illumina Human Ref-v3 v1 Expression Bead Chips and then scanned in a Bead Array reader using standard Illumina protocols (Illumina, San Diego, CA). Illumina's Bead Studio software was used for data analysis. qPCR was carried out using FastStart Universal SYBR Green Master ROX (Roche, Basel, Switzerland). Primers were designed by the Primer Express 3.0 software. Primer information is listed in **Appendix B.**

2.1.7 AlphaLISA-based H4R3me2 detection assay

Flag-WT-PRMT5 or S15A-PRMT5 and E444D mutant enzymes was purified from 293 cells using anti-Flag-M2 beads (Sigma-Aldrich, St. Louis, MO) as described in co-immunoprecipitation methods above. The enzyme prep was diluted in assay buffer (30mM Tris, pH 8.0, 1mM DTT, 0.01% BSA, 0.01% Tween-20) prior to use. SAM (New England Biolabs, Ipswich, MA) and unmethylated peptide of histone H4R3 (Anaspec, Fremont, CA) were used as the methyl group donor and PRMT5 enzyme substrate, respectively. The 23-amino acid sequence of H4R3 peptide was as follows: SGRGKGGKGLGKGGAKRHRKVLRGG-K(biotin)-NH2, with the third arginine site available for symmetric dimethylation per the assay protocol.

Acceptor beads diluted in 1X Epigenetics buffer (PerkinElmer, Waltham, MA) were added at a final concentration of 20 µg/ml to the reaction mixture and the plate was incubated at R.T. for 1 hr. Streptavidin-tagged donor beads diluted in 1X Epigenetics buffer (PerkinElmer, Waltham, MA) were then added at a final concentration of 20 µg/ml and the plate was further incubated at R.T. for 30 min. All reactions were performed in triplicate and repeated three independent times. The plates were read using an EnVision® Reader.

2.1.8 Co-immunoprecipitation assay

Cells stably expressing Flag-PRMT5 proteins were cultured to 95% confluency then lysed in co-immunoprecipitation buffer (1% Triton X-100 (v/v), 50mM Tris-HCl, pH 7.4), 150mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 20 µM aprotinin, 1 mM phenylmethanesulfonyl fluoride, and 1 mM pepstatin A) and vortexed every 4 times every 5 mins on ice. The cells were centrifuged at 3,400 rpm for 30 min, and the whole cell lysate (WCL) was transferred to the prewashed anti-Flag-M2 beads (Sigma-Aldrich,

St. Louis, MO) suspended in 1X cold PBS. The WCL/bead mixture was rotated at 4°C, overnight. Beads containing the Flag/protein complexes were then washed 4 times using 1X wash buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 1mM EDTA, 0.5% Triton X-100) followed by rotation with Flag peptide dissolved in TBS (in 10mM Tris HCl, 150 mM NaCl, pH7.4) for 1 h at 4°C to competitively elute Flag-tagged proteins from the anti-FLAG M2 beads. Eluates were then subjected to SDS/PAGE.

2.1.9 Ingenuity Pathway Analyses

Groups of genes regulated by S15A-PRMT5 were analyzed by the Ingenuity Pathway Analyses (IPA) software. The setting and filter were as follows: reference set: Ingenuity Knowledge Base (Genes _ Endogenous Chemicals); Relationship to include: Direct and Indirect; Includes Endogenous Chemicals; Filter Summary: Consider only molecules where species _ Human OR Rat OR Mouse. The *p* values for the enrichment test were calculated using Fisher's exact test, right-tailed. Log10 (p) was visualized to the left of the *p* value. $p<0.05$ was considered to be statistically significant.

2.1.10 Cell growth assay

For cell growth assays, CRC cells overexpressing Flag-PRMT5 constructs were seeded in triplicate at 2 $X10^4$ cells/well in a 6-well plate. Cells were counted at days 3, 5, 7 and 9 post-seeding using a cell hemocytometer counting chamber.

2.1.11 Boyden chamber cell migration assay

Migration assays were conducted using Boyden chambers. Briefly, a Boyden chamber consists of 8μm pore size cell culture inserts in a 24 well plate. Each insert was coated with gelatin on the side facing the lower chamber. $2X10^5$ cells were seeded in the top of the insert (upper chamber) in serum-free media while serum-rich media (10%

serum) was supplied in the well below as a chemoattractant. After 48h, migrated cells were fixed with 4% formaldehyde and stained with crystal violet. Stained cells were visualized with a light microscope at 20X magnification and quantified. Images were captured using a Canon EOS Rebel T3i Digital SLR camera.

2.1.12 Anchorage-independent growth assay

For anchorage-independent growth assays, type VII agarose (Sigma-Aldrich, St. Louis, MO) was used to prepare 2.4% and 1.2% bottom and top agar layers, respectively. 2 X 10^5 cells were resuspended in the top layer and plated onto the bottom layer. Cells were then cultured for 12-14 days at 37°C and 5% CO_2. Images of colonies were captured using a Canon EOS Rebel T3i Digital SLR camera and colony size and number were quantified using the ImageJ software (http://imagej.nih.gov/ij/).

2.1.13 Human cytokine array

Human Cytokine ELISA Array was purchased from Signosis and carried out according to the manufacturer's protocol (Signosis, San Francisco, CA). Briefly, 72hr conditioned media collected from HT29 control, HT29-WT-PRMT5, or S15A-PRMT5 stable cell lines was added to specific cytokine capture antibodies pre-coated wells for 2 h at R.T. After incubation, the wells were washed to remove unbound-labeled antibodies. The plate was further detected with HRP luminescent substrate. The level of expression for each specific cytokine was directly proportional to the emitted luminescence.

2.1.14 Chromatin immunoprecipitation-PCR assay

Cells were left untreated or stimulated with 10 ng/mL IL-1β for 1h and 4hrs and cross-linked with 1% formaldehyde for 10 min at room temperature. The cross-linking was stopped by adding glycine and cells were then washed with cold PBS, scraped, and

pelleted by centrifugation at 2000rpm. Cells were lysed in Farnham Lysis buffer [5mM PIPES pH 8.0/85 mM KCl/0.5% Tween 20] supplemented with protease inhibitors followed by chromatin shearing to yield fragments of 200–1,000 bp using a sonifier (Fisher Scientific, Hampton, NH) equipped with a microtip (40 secs on/50secs off, 4 mins at 40% power output). Sonicated lysates were centrifuged, and the resulting supernatant was diluted 5-fold with ChIP dilution buffer containing 16.7 mM Tris·HCl pH 8.1, 167 mM NaCl, 0.01% SDS, 1.1% (vol/vol) Triton X-100, and 1.2 mM EDTA. Diluted lysates were precleared for 1 h with protein A/G agarose. Immunoprecipitations were performed using ChIP-grade anti-RelA (Abcam, ab7970, Cambridge, United Kingdom) antibody at 4 °C overnight. Immune complexes were collected with protein A/G agarose, washed with low-salt wash buffer [20 mM Tris·HCl pH 8.1, 150 mM NaCl, 0.1% SDS, 1% (vol/vol) Triton X-100, and 2 mM EDTA], high-salt wash buffer [20 mM Tris·HCl pH 8.1, 500 mM NaCl, 0.1% SDS, 1% (vol/vol) Triton X-100, and 2 mM EDTA], LiCl wash buffer [10 mM Tris·HCl pH 8.1, 250 mM LiCl, 1% (wt/vol) sodium deoxycholate, 1% (vol/vol) IGEPAL-CA630, and 1 mM EDTA], and TE buffer (10 mM Tris·HCl pH 8.0 and 1 mM EDTA). Protein-DNA complexes were eluted from antibodies with elution buffer containing 1% SDS and 0.1 M NaHCO3, incubated in the presence of 192 mM NaCl for 4 h at 65 °C, and digested with proteinase K for 1 h at 45 °C. DNA was recovered using the Qiagen quick DNA purification kit and IL8 gene-specific ChIP primers (Qiagen, Hilden, Germany) were used in the PCR analyses. Primer sequences are listed in **Appendix B**.

2.1.15 Cell fractionation assay

Fractionation experiments were conducted according to the manufacturer's instructions for the nuclear extract kit (Active Motif, Carlsbad, CA). Briefly, cells were grown to about 80% confluence, washed with ice-cold PBS/Phosphatase inhibitors, collected and pelleted for 5 minutes at 500 rpm. Cell pellets were then gently

resuspended in 1X hypotonic buffer and incubated for 15 mins on ice. The cytoplasmic fraction was collected as the lysate following centrifuging for 30s at 14,000xg. The remaining cell pellet was further lysed in Complete Lysis Buffer, incubated on ice for 30 mins at 150rpm and centrifuged for 10 minutes at 14,000xg to collect the nuclear fraction. The cytoplasmic and nuclear proteins were then separated by SDS-PAGE as previously described.

2.2 Statistical analysis

Statistical analyses were performed using Prism 6 software (GraphPad, San Diego, CA). Data represent the mean ± S.D. or ±SEM as indicated. A two-tailed Student's *t*-test was used when comparing two means between groups as specified. All statistics were carried out for triplicate experiments and a $p<0.05$ was considered statistically significant.

CHAPTER 3: ASSESSING THE ROLE OF SERINE 15 PHOSPHORYLATION IN PRMT5-MEDIATED ACTIVATION OF NF-κB SIGNALING

3.1 Background and Rationale

Recently, PRMT5 has emerged as an important biomarker for several cancer types including CRC and its overexpression is highly correlated to the proliferative signaling which drives malignancy (B. Zhang *et al.*, 2015). Importantly, the cancer-associated properties of PRMT5 are mediated by its ability to methylate a variety of substrate proteins, among which are critical transcription factors that trigger the downstream signaling cascades necessary for cancer cell survival. Importantly, our previous work demonstrated that PRMT5 overexpression could substantially augment activation of the pro-inflammatory transcription factor NF-κB via methylation of R30 on its p65 subunit (Prabhu *et al.*, 2017). Moreover, approximately 75% of NF-κB–dependent genes were induced less well upon overexpression of the R30A mutant compared to the wild type p65 protein. This finding is significant, since elevated activation of NF-κB and subsequent dysregulation of its downstream target genes is one of the key mechanisms involved in promoting the malignancy of CRC and thus, PRMT5 overexpression represents an important means by which this aberrant NF-κB activity could be achieved. However, the precise mechanisms governing this PRMT5/NF-κB signaling axis are largely unknown, yet this knowledge is critical for mitigating PRMT5-mediated activation of NF-κB.

By means of mass spectrometric analysis of purified PRMT5, we identified a novel S15 phosphorylation site on PRMT5 in response to IL-1β stimulation. We therefore hypothesized that phosphorylation of PRMT5 at S15 mediates its ability to activate NF-κB signaling. Here, we investigate the significance of S15 phosphorylation using an S15A-PRMT5 mutant, with respect to its role in the transactivation of NF-κB and

induction of downstream target genes. Mechanistically, we were also prompted to perform a series of experiments to further probe the role of S15 phosphorylation in regulating the methyltransferase activity of PRMT5, complex formation between PRMT5 and p65 as well as the promoter occupancy of p65 at its classical target gene, IL8. Overall, the findings depicted in this chapter will not only enhance our understanding of the fine-tuned regulation of PRMT5, but will provide key insights into the factors underlying its specific activity towards p65, which may be of potential major significance in the context of cancers with elevated PRMT5 and NF-κB activities.

3.2 Results

3.2.1 Identification of serine 15 (S15) phosphorylation site on PRMT5

Phosphorylation of proteins remains one of the key mechanisms employed by cells to not only increase the functional diversity of the proteome but to ultimately influence various aspects of pathogenesis (Ardito *et al.*, 2017). Using mass spectrometry approaches, we screened for potential phosphorylation modifications on PRMT5. First, we overexpressed Flag-tagged PRMT5 (Flag-PRMT5) in 293IL1R cells (denoted as HEK293) that were left untreated or stimulated with 10ng/mL of IL-1β. The 293IL1R cell line was previously described by Lu *et al* (Lu *et al.*, 2010). As shown in **Figure 9**, a mass shift of 80 Da was identified on Serine 15 (S15) of the Flag-PRMT5 protein purified (using an anti–Flag-M2 antibody) from IL-1β-treated HEK293 cells, corresponding to addition of a phosphorus group on this residue.

3.2.2 Generation of stable cell lines with overexpression of Flag-WT and S15A-PRMT5

To elucidate the biological role of S15 phosphorylation, we used a PCR-induced mutagenesis approach to successfully generate an S15A-PRMT5 mutant using a previously Flag-tagged WT-PRMT5 (Flag-WT) subcloned in a pLVX-IRES-puro vector as

the template (Lu *et al.*, 2010). Next, as shown in the western blot of **Figure 10A**, we exogenously and stably overexpressed the Flag-S15A-PRMT5 mutant protein at a level comparable with Flag-WT in HEK293 cells and a panel of CRC cell lines HT29, DLD1 and HCT116 using lentiviral plasmids and a 293T packaging cell line. The resulting stable cell lines were selected under puromycin (1µg/mL). The chosen CRC cell lines are well-established representative models widely used to investigate tumor biology and biomarker discovery. Importantly, they also harbor the genetic heterogeneity of many primary colorectal tumors, further validating their utility as tools to investigate CRC biology (J. Wang *et al.*, 2017). The mutation status of the most common CRC-related genes identified in the aforementioned cell lines are outlined in **Appendix F**. In the context of this study, we overexpressed the different forms of the PRMT5 protein as a means of investigating the observable effect of modulating a single factor on a range of phenotypes. Since endogenous PRMT5 is still present in these cells, future experiments to first deplete PRMT5 using 3'-UTR shRNA approaches followed by addback of the WT and S15A-PRMT5 proteins was preferential. Although not used in the present study, the most ideal scenario would be to employ CRISPR-Cas9 knock-in of the S15A-PRMT5 at the relevant locus to avoid any potential confounding influences of endogenous PRMT5 expression. Experiments described in this and subsequent sections were conducted using a pool of early passage stable cells frozen down at -80°C and where applicable, HT29 was used as a representative CRC cell to confirm mechanistic studies carried out in HEK293 cells.

Further validation of whether S15 phosphorylation constitutes a major serine phosphorylation site on PRMT5 was conducted by immunoprecipitation of Flag-WT-PRMT5 or Flag-S15A-PRMT5 followed by western blotting with a pan serine phosphorylation (pSER) antibody. This approach was used due to unavailability of an S15 site-specific antibody. As revealed in **Figure 10B,** upon treatment of HEK293 and

HT29 with IL-1β, the immunoprecipitated Flag-WT was phosphorylated, whereas the Flag-S15A-PRMT5 mutant exhibited dramatically less serine phosphorylation, suggesting that S15 is a major phosphorylation site on PRMT5. Furthermore, alignment of PRMT5 sequences showed that this S15 site is well conserved across different species **(Figure 10C)**.

3.2.3 Phosphorylation of PRMT5 at S15 is critical for NF-κB activation

We previously discovered that overexpression of PRMT5 could dramatically enhance the methylation status of p65 and in turn NF-κB activation, whereas knockdown showed the opposite effect (Wei *et al.*, 2013). Furthermore, based on the findings thus far in **Figures 9 and 10**, we found S15 phosphorylation to be an IL-1β-inducible event and hence, we further speculated that it may potentially mediate the activation of NF-κB by PRMT5. Through a complex cascade of combinatorial phosphorylation and ubiquitination events, IL-1β acts as a potent activator of NF-κB and its related intracellular signaling events. Hence, using our established cell lines overexpressing WT-PRMT5 or the S15A-PRMT5 mutant, we assessed the activation of NF-κB using a luciferase reporter assay that involves lentiviral-based infection of our cells with a p5XIP10 construct (containing five tandem copies of the κB elements of the IP10 gene promoter upstream of the firefly luciferase reporter gene) followed by addition of the substrate, luciferin, 48 h after infection. As shown in **Figure 11**, we confirmed that overexpression of PRMT5 could significantly augment NF-κB activity in an IL-1β-inducible manner compared to the vector control (Ctrl), whereas in HEK293, HT29, DLD1 and HCT116 S15A-PRMT5 -expressing cells, NF-κB was less activated. This suggests that this phosphorylation site is important to PRMT5-mediated NF-κB transactivation and may also potentially mediate more downstream signaling effects.

Finally, we also observed that in the presence of endogenous PRMT5, the S15A-PRMT5 mutant decreased the activation of NF-κB to a level comparable or below that of the Ctrl cells, suggesting that this mutant may be acting in a dominant-negative fashion.

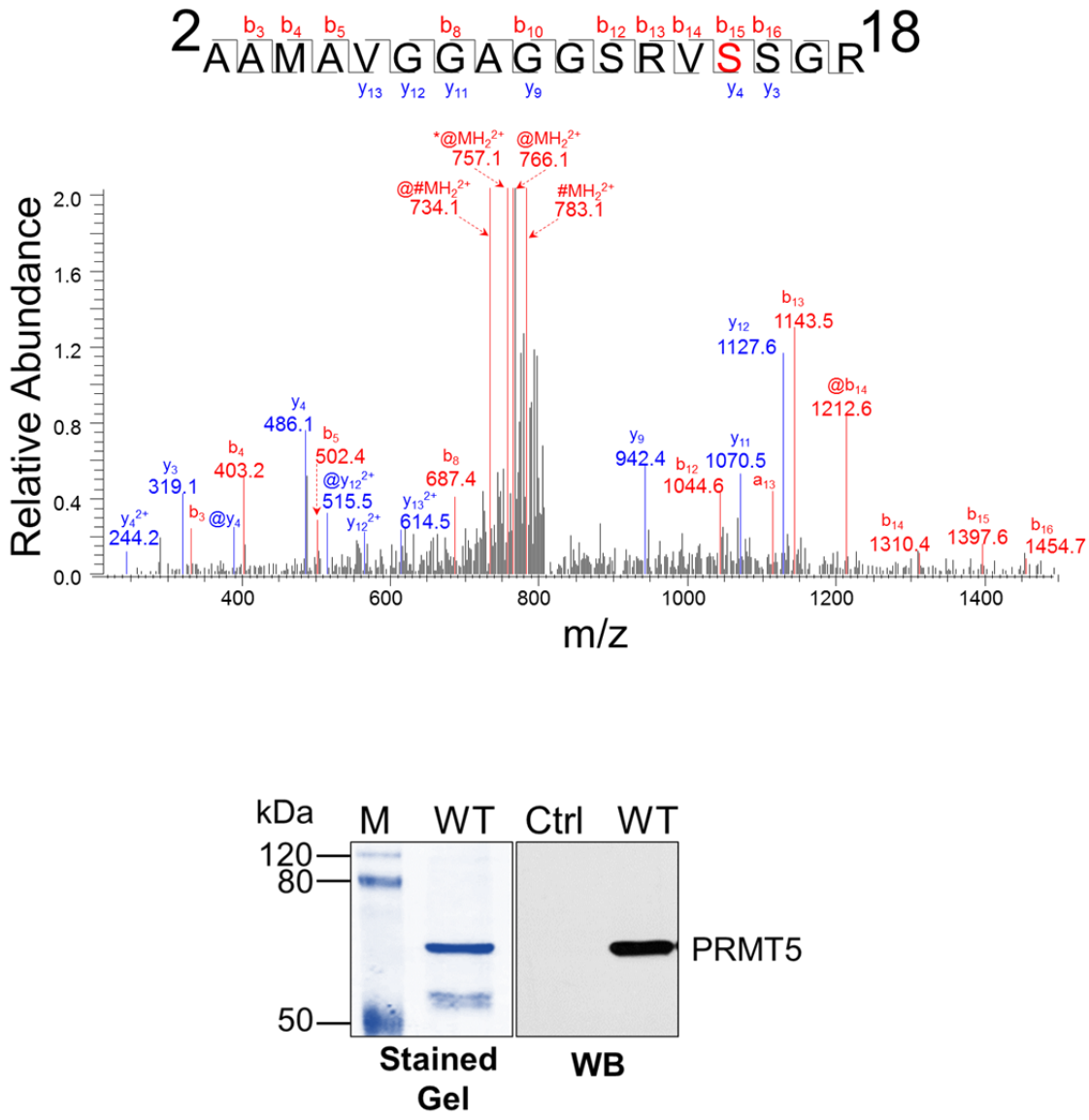


Figure 9: Identification of phosphorylation of Serine 15 (S15) on PRMT5. **Top panel,** mass spectrometry (MS) experiment identifies S15 as a phosphorylated residue in response to IL-1β treatment. A mass shift of 80Da was observed, indicating the existence of the phosphorylation modification. **Bottom panel**, Gel-code blue stained MS gel indicates a purified strong Flag-PRMT5 protein band (left). Western analysis confirmation of the identity of the purified band as PRMT5 (right).

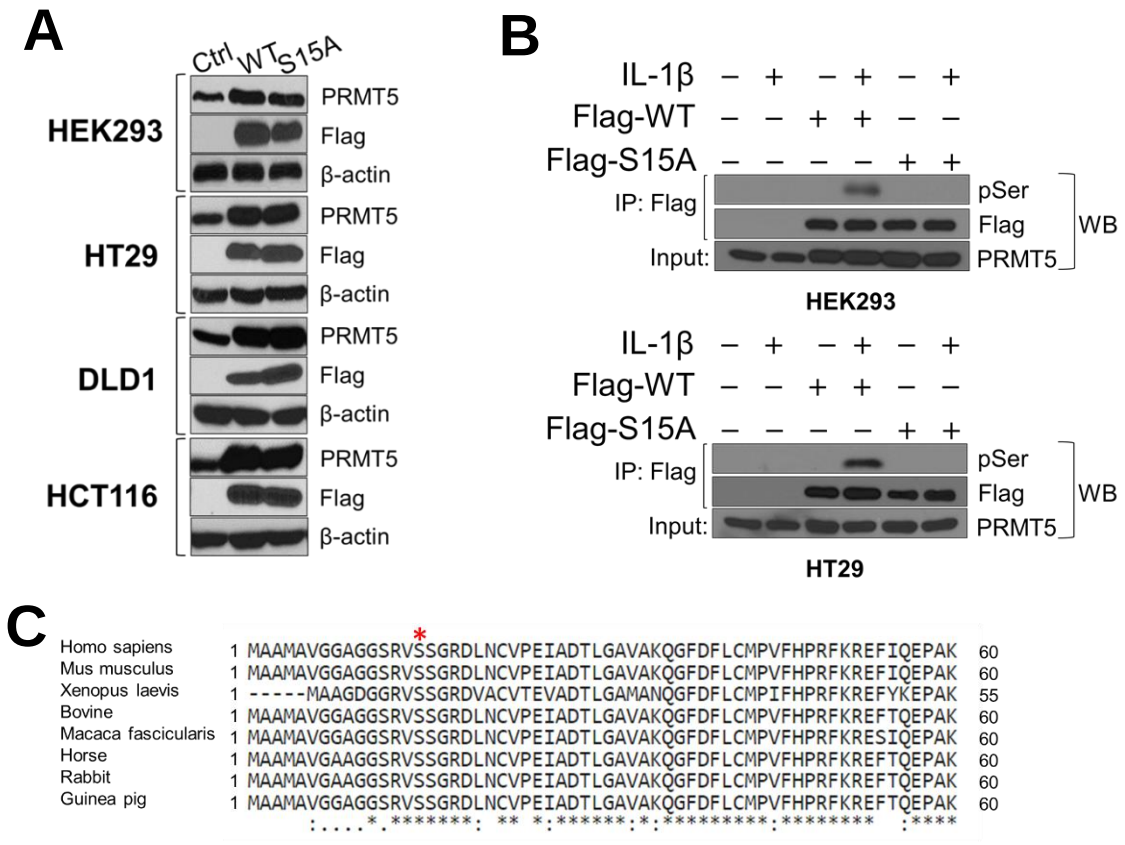


Figure 10: Establishment of wild type (WT) or Serine 15 to Alanine (S15A) mutant Flag-PRMT5 overexpressing stable cells. **A.** Western blot images, showing overexpression of Flag-PRMT5 constructs probed with anti- PRMT5, or Flag, or β-actin respectively, in HEK293 cells or HT29, DLD1 and HCT116 colon cancer cells. **B**. Confirmation of phosphorylation of PRMT5 at S15 using co-immunoprecipitation and western blot analysis. Either HEK293 (top panel) or HT29 cells (bottom panel) were treated with IL-1β or left untreated for 1 h (10 ng/mL). Samples were collected and Flag-WT-PRMT5 (Flag-WT) or Flag-S15A-PRMT5 (Flag-S15A) was further immunoprecipitated with anti-Flag-M2 beads and subjected to western analysis using an anti-phospho-serine motif antibody (pSER). The inputs were probed with anti-PRMT5 antibody. **C.** Cross-species alignment of amino acid sequences from PRMT5 proteins (residues 1-60). The conserved S15 residue is indicated on top by the red asterisk (www.uniprot.com).

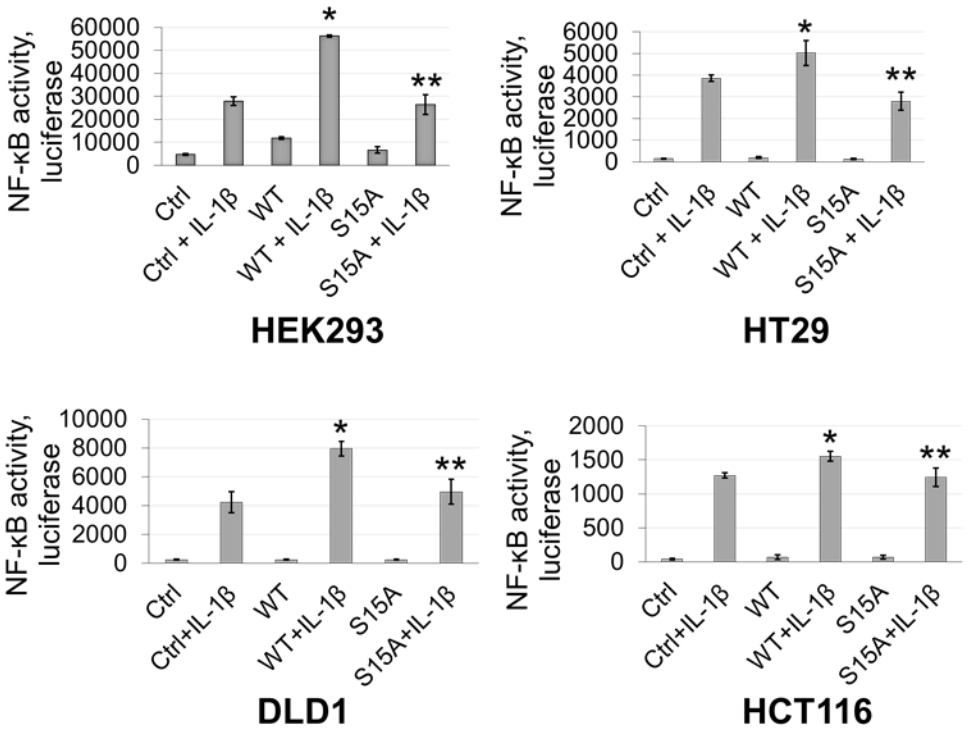


Figure 11: Phosphorylation of PRMT5 at S15 is critical for NF-κB activation in response to IL-1β. **A.** Phosphorylation of PRMT5 at S15 is critical for NF-κB activation. NF-κB luciferase assay, conducted for vector control (Ctrl), or with the stable overexpression of WT-PRMT5 (WT) or S15A-PRMT5 (S15A) in the presence or absence of 10 ng/mL IL-1β treatment in HEK293, and HT29, DLD1, HCT116 colon cancer cells. Readings were normalized to total protein concentration for respective wells. **p<0.05 vs. Ctrl+IL-1β group; **p<0.05 vs. WT+IL-1β group.*

3.2.4 Phosphorylation of PRMT5 at S15 differentially regulates a subset of NF-κB target genes

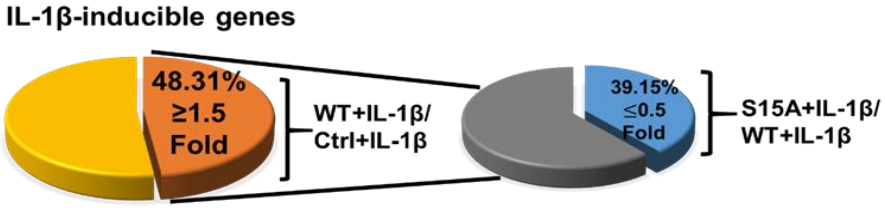
Next, we simultaneously sought to assess whether the transcriptional levels of NF-κB target genes could be upregulated by PRMT5 overexpression and compromised by the S15A-PRMT5 mutant, which are downstream events of the afore-mentioned phospho-mediated transactivation of NF-κB by PRMT5. This is also based on our previous finding in which the R30A mutant of p65 and knockdown of PRMT5 could in parallel, downregulate a significant fraction of NF-κB-dependent genes (Wei *et al.*, 2013). To initially test our hypothesis that S15A-PRMT5 would disrupt NF-κB target gene transcription, HEK293 cells with or without (Ctrl) WT-PRMT5 or S15A-PRMT5 overexpression, in the presence or absence of IL-1β were used to carry out Illumina microarray analysis. This work was done in collaboration with Dr. Pieter Faber at the University of Illinois at Chicago. We observed that of the pool of IL-1β-inducible genes, approximately 48% were further upregulated 1.5-fold or more by overexpression of WT-PRMT5 compared to the vector control (WT+IL-1β/Ctrl+IL-1β≥1.5 fold) (**Figure 12A, left pie chart**). However, when compared to these WT-PRMT5-upregulated transcripts, approximately 39% of this subset of genes failed to be upregulated in the cells overexpressing the S15A-PRMT5 mutant protein (S15A-PRMT5 + IL-1β/WT+ IL-1β≤0.5 fold) (**Figure 12A, right pie chart**).

Intriguingly, we uncovered that among the genes that were upregulated by the WT-PRMT5 but not S15A-PRMT5, were a range of cytokines (e.g., TNFα), chemokines (IL8, CCL20, CXCL10), and cell adhesion molecules (e.g., E-selectin, VCAM-1), all components implicated in CRC initiation and progression. A short list of typical genes whose induction was inhibited by the S15A mutation is shown in **Figure 12A (table)** and a full list is provided in **Appendix E**. Confirmation of the array data by qRT-PCR analysis

consistently showed that the mRNA transcript levels of candidate genes CCL20 and IL8 were further augmented by the overexpression of PRMT5 in both HEK293 and HT29 under IL-1β-stimulating conditions whereas overexpression of S15A-PRMT5 significantly attenuated this effect **(Figure 12B)**. In a cross comparison, we also found that several of these genes including IL8 and CCL20 were among those downregulated by the previously reported R30A mutation of p65 (**Table 2**), suggesting a correlation between S15 phosphorylation (of PRMT5)–and R30 methylation (of p65)–dependent gene regulation.

Collectively, these data strongly support the notion that overexpression of PRMT5 can augment the NF-κB signaling at many levels in an IL-1β-inducible and phospho-dependent fashion. Importantly, phosphorylation of S15 on PRMT5 plays a critical role in the IL-1β-inducible expression of a subset of NF-κB target genes whose functions are pro-inflammatory and cancer-associated by nature.

A



Accession No.	Gene Symbol	Fold Change		
		Ctrl+IL-1β /Ctrl	WT+IL-1β/ Ctrl+IL-1β	S15A+IL-1β/ WT+IL-1β
NM_004591.1	CCL20	471	2	0.4
NM_002089.3	CXCL2	153	2	0.4
NM_000584.2	IL8	79	2	0.5
NM_001570.3	IRAK2	3	2	0.5
NM_005204.2	MAP3K8	4	2	0.4
NM_000450.1	SELE	86	2	0.2
NM_001561.4	TNFRSF9	34	3	0.5
NM_001078.2	VCAM1	52	2	0.2

B

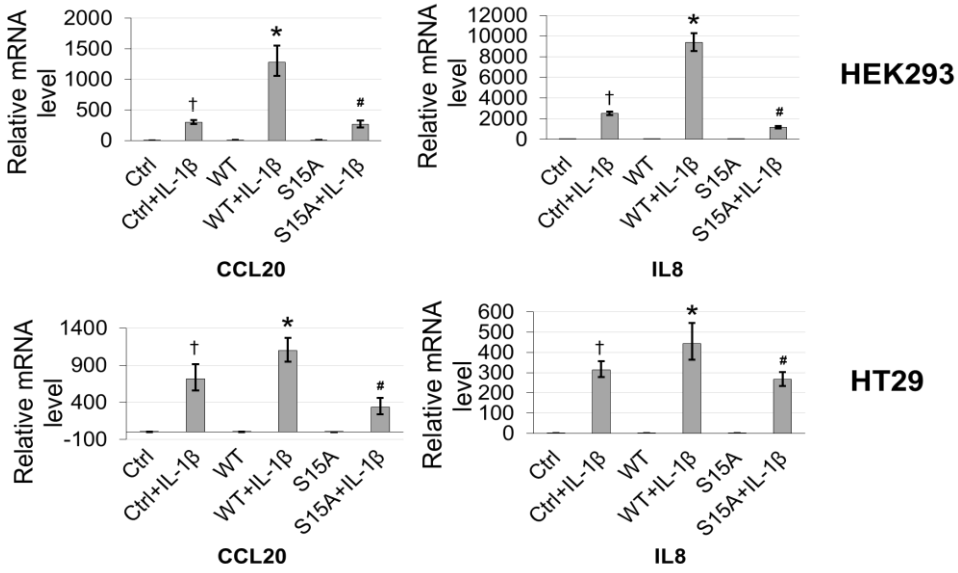
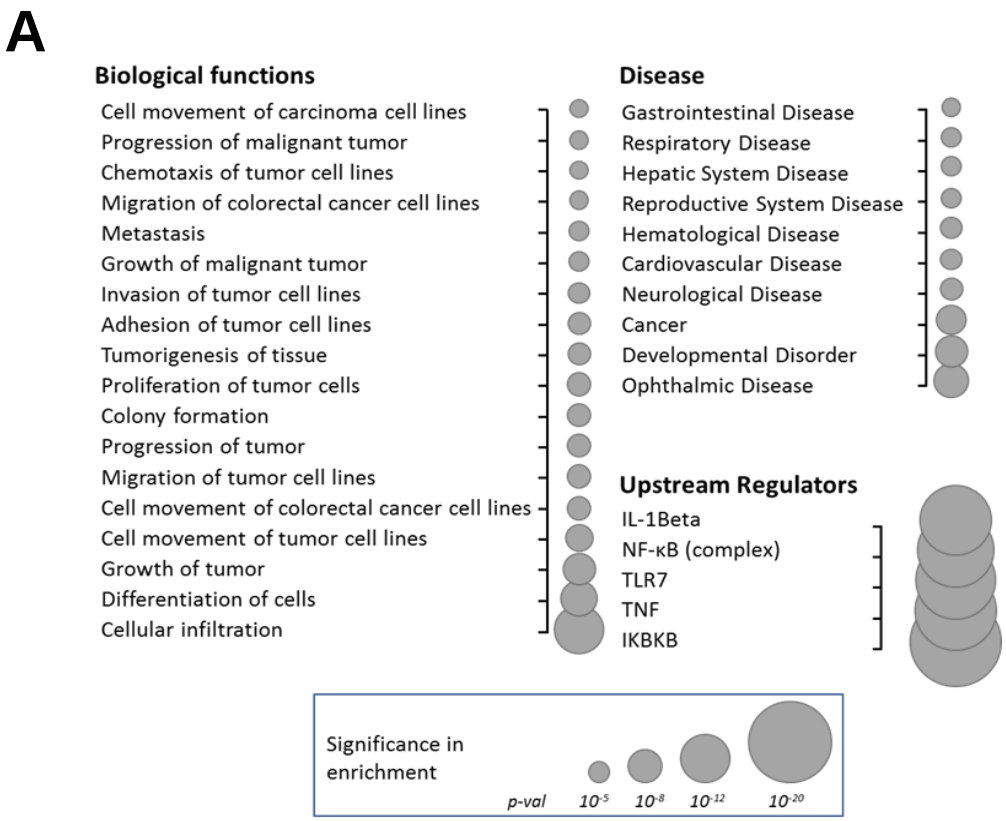


Figure 12: Phosphorylation of PRMT5 at S15 differentially regulates a subset of NF-κB target genes. **A. Top panel:** Pie-char (left, yellow and orange), representing data from human Illumiona array assay. Data indicates that upon overexpression of WT-PRMT5, the expression of 48.31% of NF-κB target genes were further augmented by >= 1.5-fold following 10 ng/mL IL-1β stimulation. Among these genes, 39.15% of genes (Pie-chart, right, gray and blue) could be downregulated by 2-fold or more (S15A + IL-1β/WT+IL-1β≤0.5) by the S15A-PRMT5 mutation. **Bottom panel:** Table, showing a short list of typical NF-κB target genes that were upregulated by WT-PRMT5 (WT) but not by the S15A-PRMT5 mutant. **B.** Confirmation of Illumina Array data with qPCR analysis, indicating relative mRNA levels of CCL20 and IL8. Ctrl: vector control cells. *$^{\dagger}p<0.05$ vs. Ctrl group; $^{*}p<0.05$ vs. Ctrl+IL-1β group; $^{\#}p<0.05$ vs. WT+IL-1β group.*

3.2.5 Top diseases and biological functions regulated by S15

To identify the signature biological functions and networks associated with the subset of genes differentially regulated by S15A-PRMT5, we further conducted an ingenuity pathway analysis (IPA) in collaboration with Drs. Guanglong Jiang and Yunlong Liu of the IUSM Center for Computational Biology and Bioinformatics. Interestingly, we observed an enrichment of terms associated with key biological functions such as “migration of tumor cells”, “proliferation of tumor cells” and “colony formation”. Moreover, networks related to “cancer” and “development disorders” were among the top enriched disease networks while IL-1β, IKBKB and the NF-κB complex were by far the most highly enriched upstream pathway regulators (**Figure 13A**). Interestingly, representative networks revealed NF-κB as a key interaction node among the genes upregulated by WT-PRMT5 and compromised by the S15A-PRMT5 mutant (**Figure 13B, Appendix D**).



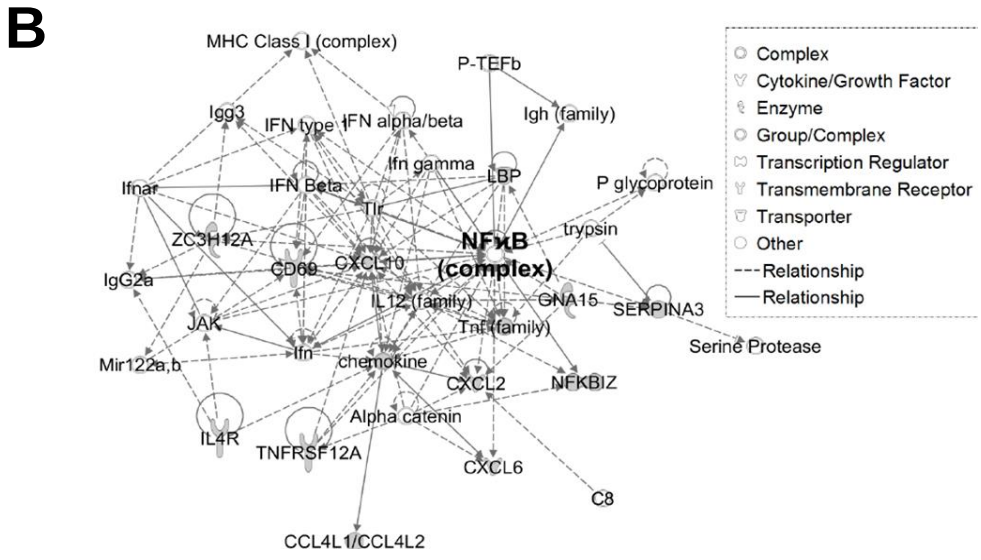


Figure 13: Top diseases and biological functions regulated by S15A-PRMT5. **A.** Subset of genes upregulated by WT-PRMT5 overexpression but downregulated by S15A-PRMT5 was used to perform Ingenuity Pathway Analysis (IPA). Enrichment results indicating top biological functions, disease networks and upstream regulators as shown in dots scaled by*–log(p).* The size of the dot shows the significant level of enrichment. **B.** IPA representative network showing genes regulated by S15A-PRMT5 with NF-κB as one of the critical nodes in this network.

Table 2: Genes commonly regulated by S15A-PRMT5 and R30A-p65

Gene Symbol	Description
CCL20	C-C Motif Chemokine Ligand 20
CXCL2	C-X-C Motif Chemokine Ligand 2
DMRT2	Doublesex And Mab-3 Related Transcription Factor 2
ESM1	Endothelial Cell Specific Molecule 1
IL32	Interleukin 32
IL8	Interleukin 8
MAFF	MAF BZIP Transcription Factor F
MAP3K8	Mitogen-Activated Protein Kinase Kinase Kinase 8
NFKBIZ	Nuclear Factor Kappa B Inhibitor Zeta
PTGS2	Prostaglandin-Endoperoxide Synthase 2
SELE	Selectin E
TM6SF2	Transmembrane 6 Superfamily Member 2
TNFAIP3	TNF Alpha Induced Protein 3
VCAM1	Vascular Cell Adhesion Molecule 1

3.2.6 S15A-PRMT5 disrupts formation of the complex between PRMT5 and p65

Post-translational modifications play pivotal roles in governing the function of proteins, in part by mediating important protein-protein interactions and the assembly of larger multi-protein complexes. We therefore hypothesized that a possible phosphoserine-dependent complex formation between PRMT5 and p65 may place it into close proximity with PRMT5, where it becomes a target for symmetric dimethylation. Hence, to further explore the possible mechanisms underlying the negative regulation of NF-κB activity by S15A-PRMT5, we wondered whether this mutant altered the PRMT5-p65 complex formation and thus could indirectly impair the downstream R30 methylation-regulated transcriptional competence of p65. As shown in **Figure 14** and consistent with our previous findings (Wei *et al.*, 2013), co-immunoprecipitation studies of Flag-WT-PRMT5 purified from cells pre-treated with IL-1β showed an enhanced complexing between PRMT5 and endogenous p65 in both HEK293 and HT29 cells. Conversely, the immunoprecipitated Flag-S15A-PRMT5 complexed less well with p65, providing the first evidence that this IL-1β-inducible PRMT5/p65 axis occurs in a phospho-dependent manner.

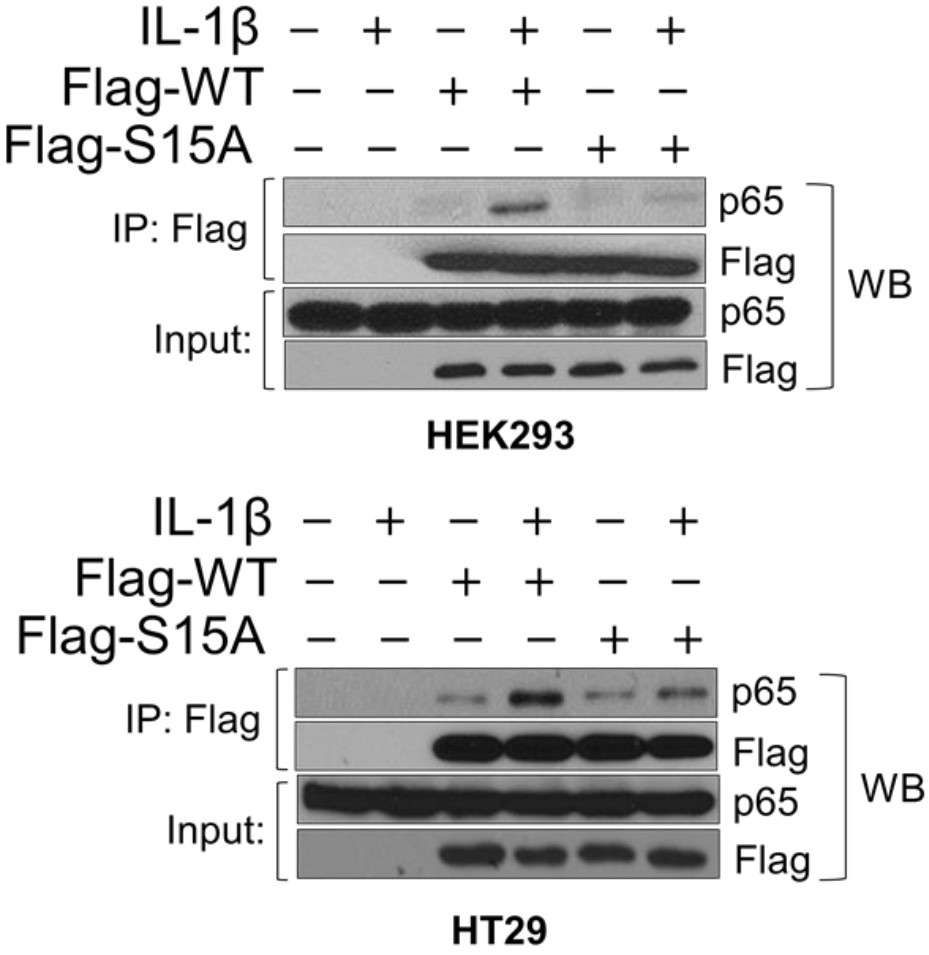


Figure 14: S15A mutant of PRMT5 disrupts its complex with p65. Co-immunoprecipitation (IP) experiments, HEK293 and HT29 cells were treated or left untreated with IL-1β (10ng/mL) for 1 h, Flag-WT-PRMT5 (Flag-WT) or Flag-S15A-PRMT5 (Flag-S15A) was immunoprecipitated with anti-Flag-M2 beads. Samples were then subjected to western blot analysis (WB) and probed with anti-p65 antibody. Inputs were probed with anti-p65 and anti-Flag antibodies.

3.2.7 S15A-PRMT5 attenuates occupancy of p65 at NF-κB target gene

Considering the positive and negative contribution of WT-PRMT5 and S15A-PRMT5 to NF-κB downstream target gene activation, respectively, we next asked whether this was associated with differential binding and occupancy of p65 at the promoter of its target genes. To provide support for this hypothesis, we were prompted to conduct ChIP-PCR analysis to determine the proximal promoter occupancy of p65 at the IL8 gene, a prototypical target gene of NF-κB that harbors a κB element in its promoter (**Figure 15A**). Moreover, this gene was also confirmed as S15A-PRMT5 - regulated using our earlier microarray and qPCR studies (**Figure 12**). Interestingly, overexpression of PRMT5 led to a strikingly enhanced occupancy of p65 at the IL8 promoter along a time course treatment with IL-1β in both HEK29 and HT29 cells **(Figure 15B)**. On the other hand, the IL-1β-induced p65 promoter occupancy was substantially reduced by S15A-PRMT5, demonstrating that S15 phosphorylation mediates the PRMT5-dependent occupancy of p65 at this important target gene.

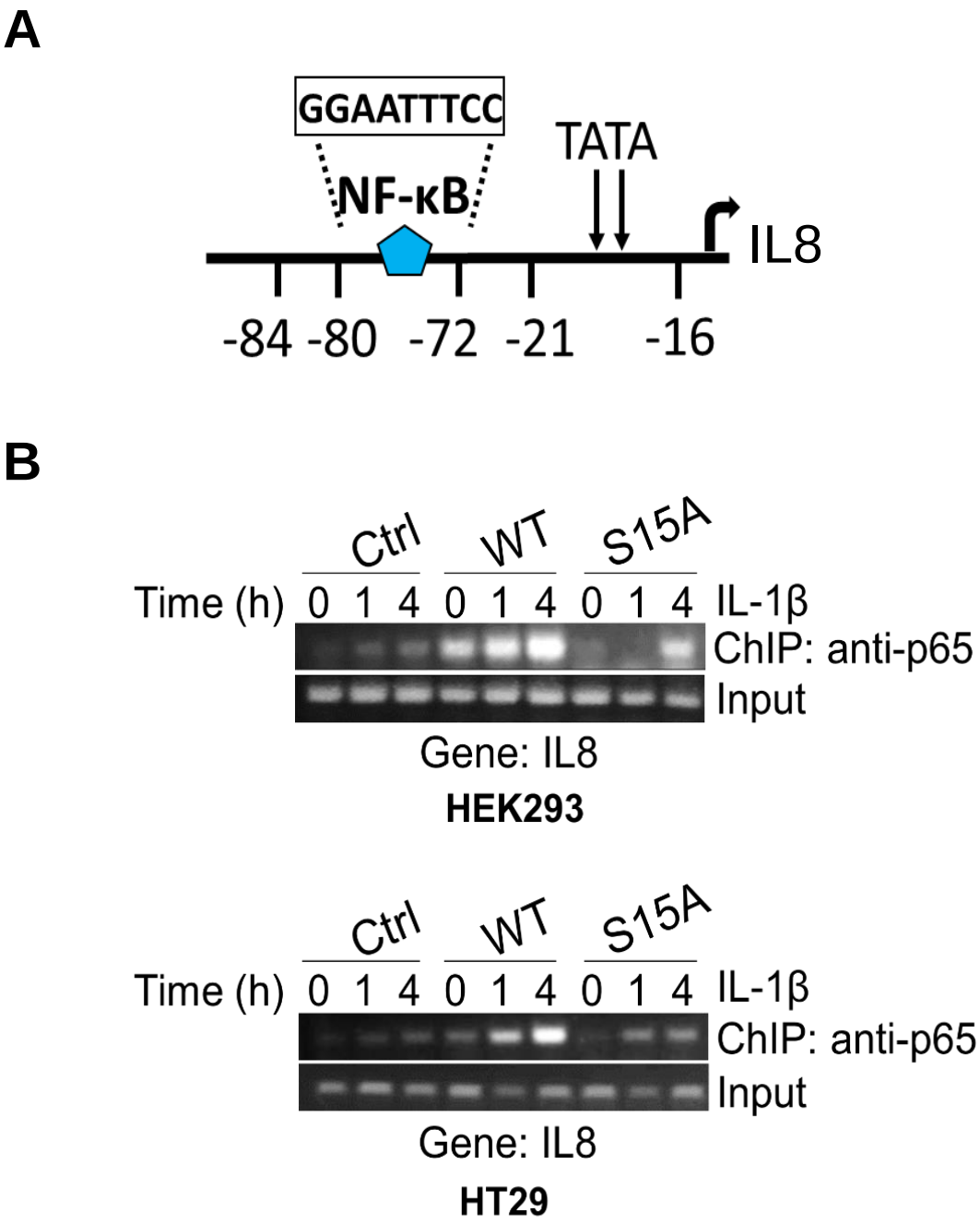


Figure 15: S15A mutant of PRMT5 attenuates occupancy of p65 at NF-κB target gene, IL8. **A.** Architecture of the IL8 promoter showing location of the NF-κB binding site. **B.** Chromatin immunoprecipitation (ChIP) assay in HEK29 and HT29 cells to detect occupancy of p65 at the typical NF-κB target gene, IL8's promoter upon IL-1β stimulation.

3.2.8 S15 phosphorylation regulates IL-1β-inducible PRMT5 methyltransferase activity

Based on the domain architecture of human PRMT5, we determined that the S15 residue is located within the unique N-terminal triosephosphate isomerase (TIM)-Barrel **(Figure 16A)**. Interestingly, several studies suggest that contributions from the TIM-Barrel may potentially control various aspects of PRMT5's function including its oligomerization state, substrate specificity and enzymatic activity. We therefore reasoned that phosphorylation of PRMT5 at S15 could influence one or more of the properties assigned to this structural region, such as its intrinsic methyltransferase activity and in turn, this could affect its activity towards NF-κB. To test this notion, we immunoprecipitated enzyme preps of Flag-WT-PRMT5 and Flag-S15A-PRMT5 from HEK293 cells that were left untreated or stimulated with IL-1β. As a negative control, we also generated and purified Flag-PRMT5 containing a glutamate-to-aspartic mutation at E444 (Flag-E444D), an invariant glutamate residue within the active site previously reported as being critical to its methyltransferase activity. Hence, Flag-E444D was used to represent an enzymatically dead mutant of PRMT5. We then employed our previously described AlphaLISA assay (Prabhu *et al.*, 2017) to compare the specific activity of Flag-WT, Flag-S15A-PRMT5 and Flag-E444D using an H4R3 peptide as a substrate.

As shown in **Figure 16B**, IL-1β treatment significantly enhanced the activity of WT-PRMT5 while S15A-PRMT5 showed significant attenuation of this ligand-induced activity. Intriguingly, there was no significant difference between the activity of the WT and S15A-PRMT5 enzymes under basal conditions, suggesting a putative IL-1β-dependent mechanism by which S15 phosphorylation induces conformational changes in such a way to enhance PRMT5's activity albeit other factors are also likely at play since this enzymatic activity was not completely abolished by S15A-PRMT5. One limitation of the AlphaLISA technique is that the acceptor beads used in the assay are conjugated to an antibody that specifically recognizes the H4R3me2s mark and so while

ideally, we would have liked to use p65 as a substrate for this experiment, the necessary reagents were not readily available. Alternatively, we collaborated with Genescript to generate a customized antibody that recognizes R30 symmetric dimethylation of p65 (p65-R30me2s). Since differential PRMT5 activity could impact the R30me2s status of p65, we conducted western blot analyses to detect this methylation in WT-PRMT5 versus S15A-PRMT5 -overexpressing HEK293 cells. As predicted, we observed that the S15A-PRMT5 -overexpressing cells showed less IL-1β-inducible p65-R30me2s levels compared to the WT (**Figure 16C**), further reiterating the critical role of S15 phosphorylation in regulating the activity of PRMT5 towards p65.

3.2.9 S15A-PRMT5 does not alter the subcellular localization of PRMT5

Phosphorylation has been frequently shown to regulate the localization of modified proteins. We therefore wondered whether the reduced PRMT5/p65 complex formation and attenuated transactivation of p65 conferred by the S15A-PRMT5 mutant may be due to other factors such as altered subcellular localization of S15A-PRMT5 compared to WT-PRMT5. We therefore carried out cell fractionation assays to determine the impact of the S15A-PRMT5 mutant on the cytoplasmic and nuclear localization of PRMT5. Qualitatively, no appreciable difference in the subcellular compartmentalization pattern of the WT-PRMT5 and S15A-PRMT5 proteins was observed, indicating that the cytoplasmic-nuclear distribution of PRMT5 was not impacted by this modification **(Figure 17A)**.

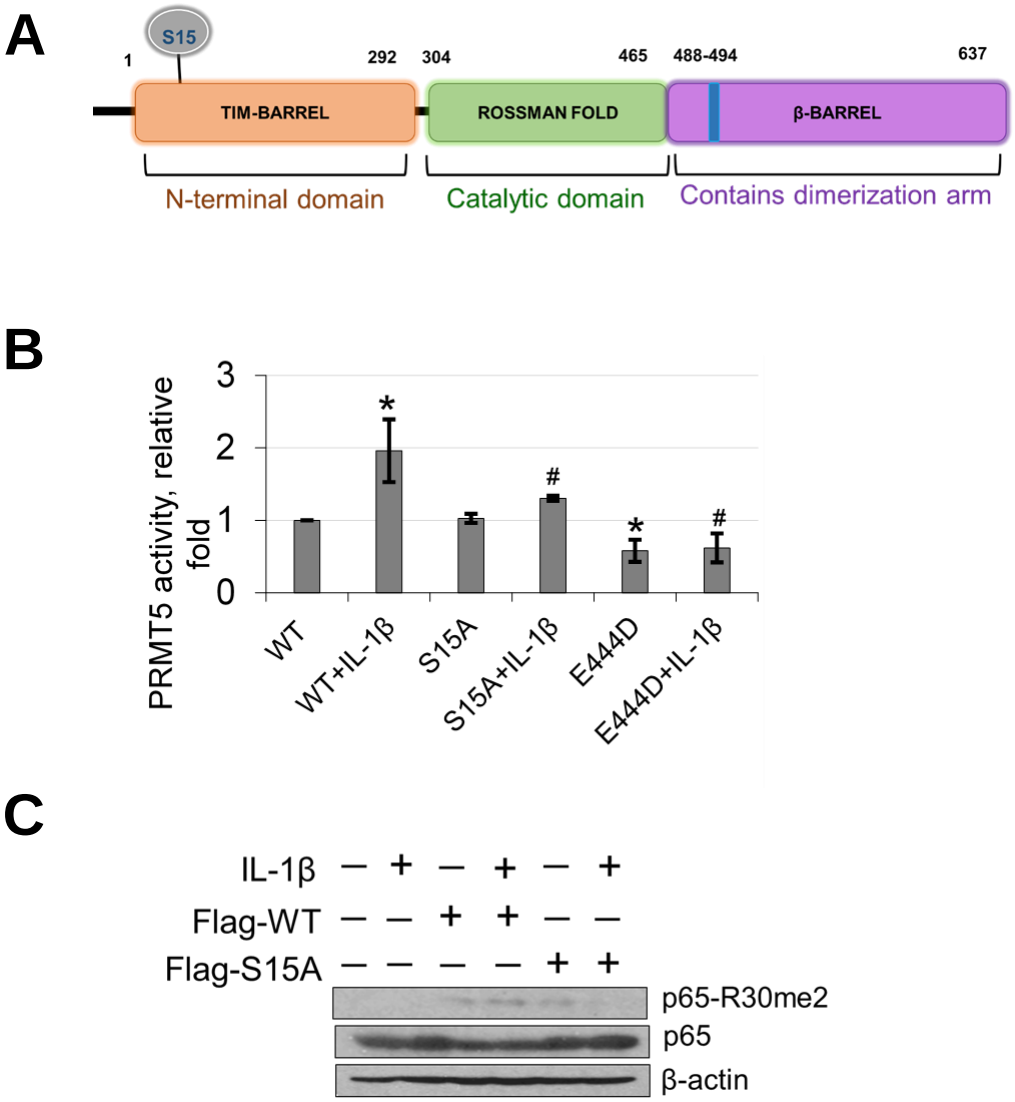


Figure 16: S15 phosphorylation is located within the TIM barrel domain of PRMT5 and regulates IL-1β-inducible PRMT5 methyltransferase activity. **A.** Overview of the domain architecture of human PRMT5. The S15 residue is located in the unique N-terminal TIM-Barrel, which might be critical for PRMT5 substrate binding. **B**. S15 phosphorylation is critical for the methyltransferase activity of PRMT5. AlphaLISA assay was conducted by using biotinylated histone H4 as a PRMT5 substrate. Graph shows detection of specific methyltransferase activity of WT-PRMT5 (WT) or S15A-PRMT5 (S15A) mutant enzymes purified from HEK293 cells in the presence or absence of IL-1β (10ng/mL) treatment. E444D-PRMT5 (E444D) was used as an enzymatic dead mutant control. S-adenosyl methionine (SAM) was used as the methyl donor for the reaction. The data represent the means ± standard deviation (S.D.) for three independent experiments. **p<0.05 vs. WT group; #p<0.05 vs. WT+IL-1β group.* **C.** Western blot image, showing symmetric dimethylation of arginine 30 of p65 (p65-R30me2) in Flag-WT-PRMT5 and Flag-S15A-PRMT5 in the presence or absence of IL-1β (10ng/mL) treatment. Blot was probed with anti-p65-R30me2, anti-p65, or β-actin respectively, in HEK293 cells.

3.2.10 S15A-PRMT5 acts independently of IκBα degradation and p65 nuclear translocation

In resting cells, the p65/p50 heterodimer of NF-κB is retained in the cytoplasm through its complex with IκBα. Upon activation with stimuli such as IL-1β, canonical NF-κB signaling involves various critical steps including the phosphorylation, ubiquitination, and subsequent proteasomal degradation of IκBα, which leads to the nuclear translocation of the p65/p50 subunits followed by DNA binding, and gene transcription. We therefore speculated that S15A-PRMT5 could alter the degradation of IκBα in response to IL-1β and thus potentially impair p65 translocation to the nucleus. To test this possibility, we treated HEK293 cells with a timecourse of IL-1β wherein we would expect rapid ligand-induced IκBα degradation which eventually becomes resynthesized. As shown in **Figure 17B**, no significant difference in the IκBα degradation pattern was observed between the control, WT-PRMT5- and S15A-PRMT5 -expressing cells. Moreover, no qualitative difference in the translocation of p65 to the nucleus was detected in our fractionation experiments conducted using the same cells (**Figure 17A**), indicating that S15A-PRMT5 may be acting independently of these mechanisms. To provide a more complete picture, future experiments to determine the effect of the R30A mutation on the localization of p65 are also warranted.

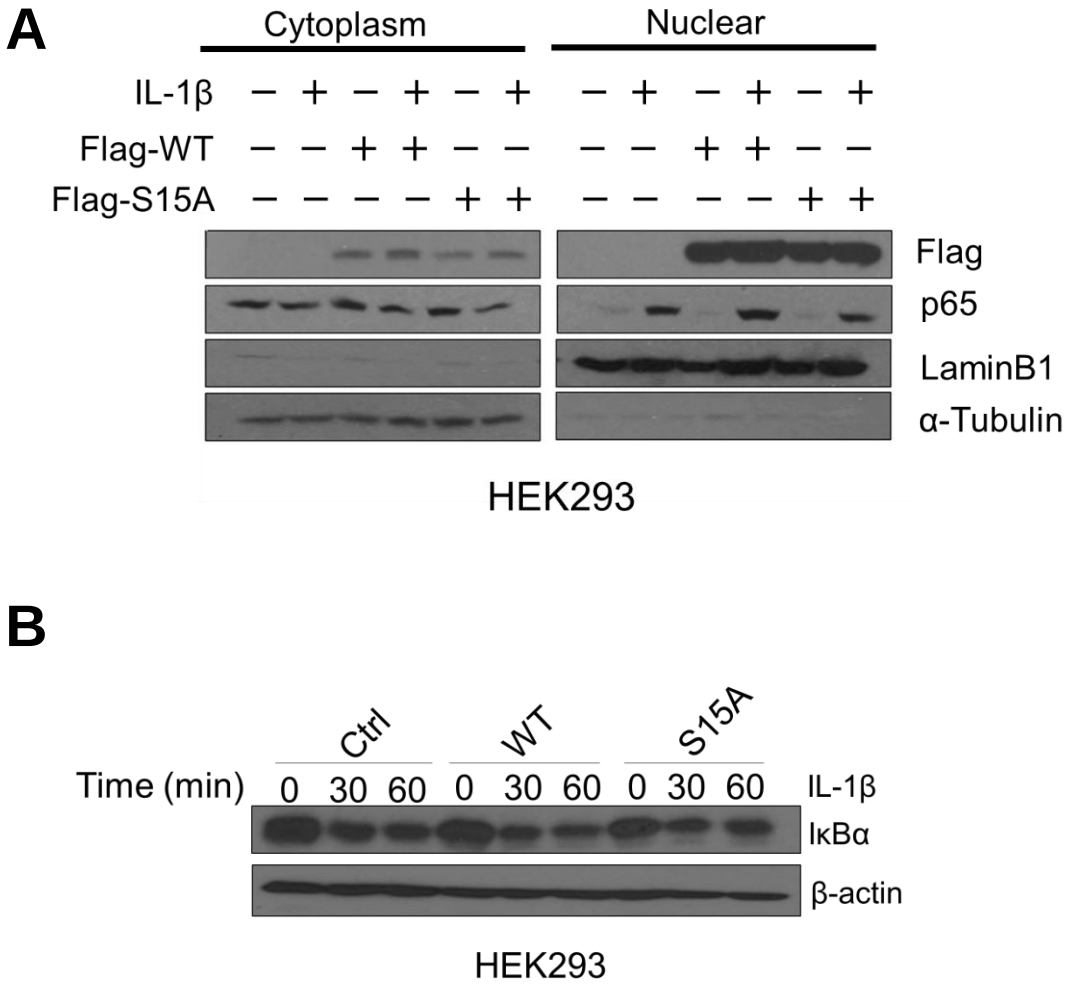


Figure 17: S15A-PRMT5 does not alter subcellular localization of PRMT5 and acts independently of IκBα degradation and p65 nuclear translocation. **A.** Cell fractionation assay, showing subcellular compartmentalization of Flag-PRMT5 in HEK293 cells. Western blot was probed with anti-Flag, p65, LaminB1 and α-tubulin antibodies. **B.** Western blot, showing IL-1β-induced IκBα degradation pattern in control, WT-PRMT5 (WT) and S15A-PRMT5 HEK293 cells.

3.3 Summary and Discussion

A wealth of reported findings supports the essential biological functions of PRMT5 in a plethora of cellular processes including proliferation, transcriptional activation/repression, signal transduction and cell differentiation. Due to its important role in these processes, PRMT5 has emerged as a critical biomarker for a variety of human cancers, including CRC and its frequent overexpression is implicated in driving cancer signaling, growth and survival (Shailesh *et al.*, 2018). The cancer-associated properties of PRMT5 are mediated by its symmetric dimethylation of both histone and non-histone substrates which in turn trigger the downstream oncogenic signaling cascades necessary for malignancy. Unfortunately, little is known about the underlying mechanisms that fine-tune PRMT5's activity and/or involvement in these signaling axes, yet this knowledge is critical since these mechanisms form the basis for devising rational therapeutic strategies for targeting PRMT5. In this regard, accumulating studies demonstrate that PRMT5 can be regulated by posttranslational modifications such as phosphorylation.

In the present study, we report a novel S15 phosphorylation event on PRMT5 in response to IL-1β stimulation. Based on our previous discovery that overexpression of PRMT5 plays a role in enhancing IL-1β-induced NF-κB-mediated transcriptional responses through R30 methylation of the p65 subunit of NF-κB, we postulated that this phosphorylation event may positively regulate this important PRMT5/NF-κB signaling axis. First, we found that S15 phosphorylation was important for the IL-1β-inducible transactivation of p65 by PRMT5 and downstream activation of a subset of NF-κB target genes associated with distinct cancer-related gene networks. Second, we established that mechanistically, the S15A-PRMT5 mutant decreased the activation of NF-κB signaling by attenuating the complex between PRMT5 and p65 and compromising the promoter occupancy of p65 at its target gene, IL8. Moreover, overexpression of S15A-

PRMT5 correlated with reduced R30 methylation of p65, suggesting that S15 phosphorylation may act as a crucial intermediate for the enhanced NF-κB response observed with PRMT5 overexpression. Third, using an *in vitro*-based AlphaLISA assay, we uncovered for the first time that IL-1β could enhance the methyltransferase activity of PRMT5 which was reduced by the S15A-PRMT5 mutant. These findings support a complex model of the regulation of PRMT5 by serine phosphorylation that define both methyltransferase activity, protein-protein interactions as well as expression of NF-κB-regulated gene signatures.

Phosphorylation is a potent regulatory mechanism of protein function and is arguably the most commonly studied PTM. In eukaryotes, it is a highly dynamic process that involves the kinase-catalyzed covalent attachment of a negatively charged phosphate group to mainly serine, threonine and tyrosine residues, which can be removed by the action of phosphatases. Importantly, this PTM significantly modifies the function of proteins by inducing conformational changes and offers a dynamic way to regulate subcellular localization, protein stability and enzymatic activity. Additionally, it can mediate interactions between the phosphorylated protein and a range of binding partners, thus generating phospho-dependent protein signaling networks. Oftentimes, these phospho-dependent interactions are signal-induced. Here, we identified the IL-1β-inducible phosphorylation of PRMT5 at S15 as positively modulating its complexing with p65. Considering the spectrum of PRMT5 target proteins and binding partners, the factors driving its interaction with one protein or another is innately complex and contextual. However, it is reasonable to consider that S15 phosphorylation may act to either confer discriminatory specificity towards p65 or trigger its complex with other substrates or even other proteins of the PRMT5 interactome in a context- and perhaps cell-type-dependent manner. In fact, recent evidence suggests that phosphorylation of PRMT5 may endow specificity in its interactions. For instance, Espejo *et al*

demonstrated that phosphorylation of threonine sites within the C-terminal tail of PRMT5 favored a phospho-dependent interaction with 14-3-3 over PDZ-domain-containing proteins, thus generating an interaction switch (Espejo *et al.*, 2017). Furthermore, several reports suggest that among the larger multimeric complexes that PRMT5 associates with, are various cofactors and adaptor proteins such as MEP50/COPR5 and RioK1, respectively. Under different contextual clues, these interacting partners can determine the substrate selection of PRMT5 such as its preferential methylation of histones versus other target substrates. Hence, further studies to elucidate whether S15 phosphorylation is important for PRMT5's interaction with other cofactors, adaptor proteins or substrates beyond p65 are warranted.

Other PTMs have been shown to influence other aspects of PRMT5 function, including its oligomerization state and methyltransferase activity. Nie *et al* showed that CARM1-mediated asymmetric dimethylation of PRMT5 enhanced its methyltransferase activity by promoting its dimerization (Nie *et al.*, 2018). Similarly, Lattouf *et al* identified LKB1-mediated threonine phosphorylation as being important in enhancing its activity by regulating its cofactor protein interaction (Lattouf *et al.*, 2019). Interestingly, the work outlined in this chapter is of great import in that it has established a novel mechanism of enhancing PRMT5 methyltransferase activity via IL-1β-mediated serine phosphorylation which constitutes another layer of unique contribution to the field. The significance of this finding is further reinforced by the critical link between IL-1β-mediated S15 phosphorylation, enhanced PRMT5 activity and increased NF-κB activation. IL-1β is frequently secreted by cells in the tumor microenvironment and serves as a potent activator of pro-inflammatory signaling. Hence, our data supports a model in which this PRMT5/NF-κB signaling node is activated by IL-1β to potentially promote inflammation and tumor invasiveness. In the future, we would like to further explore this exciting possibility using physiologically relevant and well-defined tumor microenvironment

models. It is also important to acknowledge that other cytokines and signaling molecules such as TNF-α can activate NF-κB and thus we cannot rule out the possibility that these molecules could also induce phosphorylation of PRMT5 and thus converge on PRMT5/NF-κB signaling. Future studies are needed to tease out these intricacies.

Recent knowledge regarding the known structural features of PRMT5 may provide critical insight into the catalytic and protein-protein interacting regulatory roles of S15 phosphorylation. Like other PRMTs, PRMT5 contains a catalytic domain, the Rossmann-fold, which interacts with the methyl donor SAM and is highly conserved between the *Caenorhabditis elegans* (*C. elegans*), *Xenopus laevis* (*X. laevis*) and Human isoforms of PRMT5. At the C-terminus is the β-barrel which contains a dimerization domain important for the formation of multimeric complexes. At the N-terminus is the TIM barrel, which is unique to PRMT5. This domain has been primarily implicated in promoting PRMT5 oligomerization and recruitment of cofactors such as MEP50 (S. Antonysamy, 2017). Additionally, the TIM barrel serves as a scaffold for the binding of adaptor proteins, such as pICLn and RioK1, which dictates whether PRMT5 methylates Sm proteins or nucleolin, respectively (Guderian *et al.*, 2011). Hence, this domain is important for the assembly of PRMT5 complexes and their subsequent substrate specificities. Intriguingly, recent studies provide new evidence that the TIM barrel is also critical to regulating the catalytic activity of PRMT5. The catalytic activity of a mutant human PRMT5 lacking the N-terminal region encompassing the TIM-barrel domain was shown to be severely compromised, suggesting that the TIM-barrel domain has other essential functions in addition to being important for PRMT5 dimerization and substrate selectivity (L. Sun *et al.*, 2011). This is consistent with other reports that demonstrated that residues within the TIM barrel of several TIM barrel-containing enzymes were involved in the metal-ligation and phosphate-binding essential for catalysis (Nagano *et al.*, 2002). Taken together, these findings have potentially

significant implications since the S15 residue is located within this TIM barrel region. This argues a possible regulatory role for S15 phosphorylation within this segment based on our preliminary evidence that this PTM may potentially affect some of the cellular and biochemical properties of PRMT5. So far, this is consistent with our data that shows the ability of this PTM to regulate the IL-1β-inducible catalytic activity of PRMT5 which coincided with its complexing with, and subsequent dimethylation of p65 at R30.

Finally, we elegantly showed that compared to the WT-PRMT5, overexpression of S15A-PRMT5 -PRMT5 correlated with reduced occupancy of p65 at the IL8 promoter which mechanistically explains the attenuated expression of IL8 previously observed by microarray and qPCR analyses. We recognize that since p65 occupancy was not completely abrogated by the S15A-PRMT5 -PRMT5 mutant, we can infer the influence of S15 phosphorylation-independent mechanisms in mediating the recruitment of p65 to cognate promoter sequences. Moreover, future ChIP experiments are needed to determine if the afore-mentioned phenomenon is observed at other prototypical NF-κB target genes other than IL8. Finally, to corroborate our findings and provide further support for a phosphoPRMT5-mediated regulation of R30 p65 methylation model, it would also be interesting to see if we observe a similar reduction in the occupancy of p65 at the IL8 promoter in cells expressing the R30A mutant of p65 or with knockdown of PRMT5.

3.4 Concluding Remarks

In summary, this chapter is based on the hypothesis that phosphorylation of PRMT5 at S15 regulates the dimethylation, transactivation and target gene expression of NF-κB by facilitating phospho-dependent methyltransferase activity and formation of a complex between PRMT5 and p65. Moreover, it has become increasingly clear that PRMT5 is phosphorylated at several residues, including S15. However, whether these

modifications act as a universal or cell-type dependent regulatory phenomenon is still unknown and warrants further investigation. Finally, our discovery of this novel phospho-dependent PRMT5 interaction with p65 suggests that crosstalk between kinases and PRMTs may play a pivotal role in modulating the diverse cellular functions of PRMT5.

CHAPTER 4: ASSESSING THE FUNCTIONAL ROLE OF SERINE 15 PHOSPHORYLATION OF PRMT5 IN CRC CELLS

4.1 Background and Rationale

The multifaceted role of PRMT5 within many biological contexts naturally causes its deregulation to be implicated in a variety of cancers, including CRC. Although inhibition of PRMT5 holds great therapeutic potential, there is still a significant knowledge gap concerning the underlying mechanisms that contribute to its cancer-associated properties. In this regard, our discovery that PRMT5 is regulated by S15 phosphorylation and that this distinct PTM is essential to its activation of the major pro-survival NF-κB pathway is highly significant. Sustained activation of NF-κB is observed in 50-60% of CRC cases, and is increasingly recognized as a crucial player in many of the steps of disease progression and thus, phospho-dependent PRMT5-mediated activation of NF-κB represents one potential mechanism utilized by cancer cells to maintain growth and survival. We provided further support for this notion in a recent study from our lab which revealed that along with its established role in augmenting NF-κB signaling, overexpression of PRMT5 could substantially enhance the anchorage-independent growth, proliferative and migratory capabilities of CRC cells while knockdown had the opposite effect (Prabhu *et al.*, 2017).

Interestingly, our IPA data (**Chapter 3**) also revealed that genes significantly augmented by PRMT5 overexpression but compromised by S15A-PRMT5 were associated with an enrichment of the terms “migration of tumor cells”, “proliferation of tumor cells” and “colony formation”. Collectively, this led us to hypothesize that the S15A-PRMT5 mutant protein could in part, impair the tumor-promoting functions exerted by PRMT5 in CRC. In this chapter, we investigate the functional significance of phosphorylation of PRMT5 at S15 in the context of CRC as it relates to these typical cancer phenotypes. The work presented here will provide key insight into one

potential mechanism by which PRMT5 contributes to CRC, with implications for other cancer types with elevated PRMT5 expression and/or activity.

4.2 Results

4.2.1 S15 phosphorylation mediates CRC cell growth associated with overexpression of PRMT5

Compared to their benign counterparts, unchecked proliferation is a hallmark feature of cancer cells (Fouad *et al.*, 2017). Previously, we showed that overexpression of PRMT5 could significantly enhance the proliferative capabilities of CRC cells while knockdown or pharmacological inhibition of PRMT5 had the opposite effect (Prabhu *et al.*, 2017). Moreover, our IPA data suggested that S15A-PRMT5 -downregulated genes were implicated in proliferation-associated biological functions. To test the effect of the S15A-PRMT5 mutant on CRC cell growth, cells overexpressing the WT- or S15A-PRMT5 proteins were seeded in triplicate in 6-well plates and assessed for growth over a period of 9 days of culture. As shown in **Figure 18**, overexpression of WT-PRMT5 had a growth-enhancing effect compared to the control, recapitulating our previous findings. Conversely, overexpression of S15A-PRMT5 attenuated growth compared to WT, strongly suggesting that the enhanced proliferative capacity of CRC cell associated with overexpression of PRMT5, is at least in part, a phospho-mediated process.

4.2.2 S15 phosphorylation mediates CRC cell anchorage-independent growth associated with overexpression of PRMT5

Cancer-derived cells are able to survive and form colonies suspended in agar in the absence of anchorage to the extracellular matrix (ECM) and neighboring cells, a phenomenon termed anchorage independence of growth (Stoker *et al.*, 1968). This unique property of cancer cells has been routinely used to identify tumors with invasive

and metastatic potential since it presumably reflects the tendency of tumor cells to survive and grow in inappropriate locations *in vivo* (Mori et al., 2009). Here, we tested whether the S15A-PRMT5 mutant could affect the extent of colony formation in CRC cell lines. WT-PRMT5-overexpressing cells showed a significant increase in both the size and number of colonies formed whereas the S15A-PRMT5 -overexpressing cells formed less and significantly smaller colonies **(Figure 19),** confirming that the role of PRMT5 in promoting anchorage-independent CRC cell growth is at least in part, dependent on S15 phosphorylation.

4.2.3 S15 phosphorylation mediates CRC cell migration associated with overexpression of PRMT5

Compared to normal cells, cancer cells have an increased ability to migrate, another feature vital for tumor invasion and metastasis (Fouad *et al.*, 2017). To validate the role of S15 phosphorylation in regulating the migratory potential of CRC cells, we used a Boyden chamber consisting of 8μm pore size cell culture inserts in a 24 well plate (H. C. Chen, 2005). Control, WT or S15A-PRMT5 -overexpressing cells were seeded in the top of the insert in serum-free media while serum-rich media was supplied as a chemoattractant to lower chamber. After 48h, migrated cells were fixed, stained with crystal violet, visualized and counted. Consistent with previous findings, the overexpression of WT-PRMT5 resulted in a significant increase in the number of migrated cells whereas overexpression of the S15A-PRMT5 mutant had the opposite effect **(Figure 20)**, implicating S15 phosphorylation as being critical to the tumor invasive functions exerted by PRMT5.

4.2.4 S15A-PRMT5 compromises secretion of cytokines and chemokines

Within the tumor microenvironment, pro-inflammatory factors secreted by cancer and immune cells serve as important factors to promote the tumor stemness, proliferation invasion and migration necessary for disease progression (Setrerrahmane *et al.*, 2017). Growing evidence suggests that PRMT5 plays an important role in regulating the expression of a number of these factors including several cytokines and chemokines (Harris *et al.*, 2014; Harris *et al.*, 2016; Richard *et al.*, 2005). Moreover, from our microarray data (**Chapter 3**), we also found that many of these factors are among the NF-κB target genes regulated by phosphorylation of PRMT5 at S15. To test the effect of S15A-PRMT5 on the secretion of these factors, we collected conditioned media from HT29 cells overexpressing the WT- or S15A-PRMT5 protein. We then used a Human Cytokine ELISA Array to determine the relative levels of a panel of secreted cytokines, chemokines and growth factors.

Compared to the control and WT-PRMT5-overexpression counterparts, S15A-PRMT5 cells compromised the secretion of a fraction of the cytokines/chemokines/growth factors tested, with some factors showing a more dramatic reduction than others (**Figure 21**). This further supports the notion that phosphorylation of PRMT5 at S15 regulates a subgroup of genes, among which are several confirmed NF-κB target genes including TNFα, interferon gamma-induced protein 10 (IP-10) and interleukin-4 (IL-4). Importantly, the upregulation of these cytokines has been linked to modulating inflammation, cancer cell proliferation, migration and angiogenesis, lending further credence to the role of S15 phosphorylated of PRMT5 in partially facilitating some of functions of PRMT5 in these processes.

Taken together, these data demonstrate the important role of S15 phosphorylation in regulating the cancer-associated functions exerted by PRMT5. This knowledge has provided deeper insight into some of the underlying mechanisms

involved in deregulation of PRMT5 and its potential link to CRC malignancy. One of our long-term goals is to translate our findings into a powerful diagnostic for identifying higher-risk patients based on molecular evidence supporting hyperactivation of this phospho-regulated PRMT5/NF-κB signaling node and into a survival benefit for patients. To facilitate this, future efforts to develop a highly specific antibody that recognizes the S15 phosphorylation mark in patient tissue samples will be necessary.

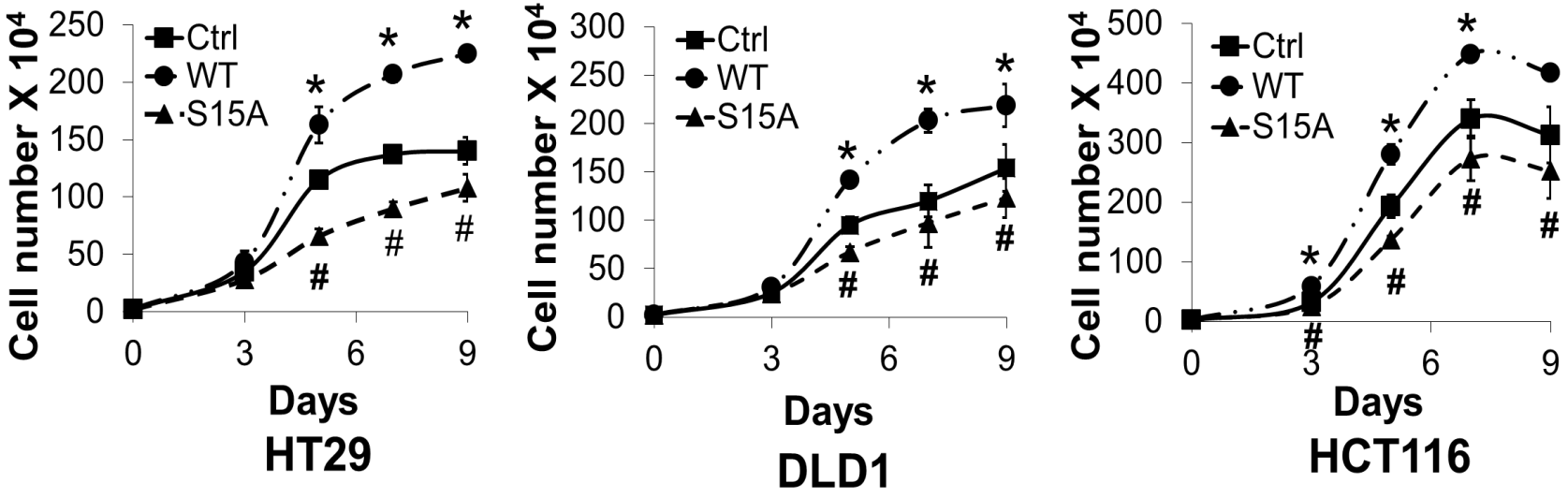


Figure 18: S15 phosphorylation mediates CRC cell growth associated with PRMT5 overexpression. Cell growth assay compares cell numbers of vector Ctrl, WT-PRMT5 (WT) or S15A-PRMT5 (S15A) mutant-overexpressing cells in HT29, DLD1 and HCT116 colon cancer cells. $2X10^4$ cells were seeded and counted using a cell counting chamber at days 3, 5, 7 and 9. The data represent the means ± standard deviation (S.D.) for three independent experiments. **p<0.05 vs. Ctrl group; $^{\#}$p<0.05 vs. WT group.*

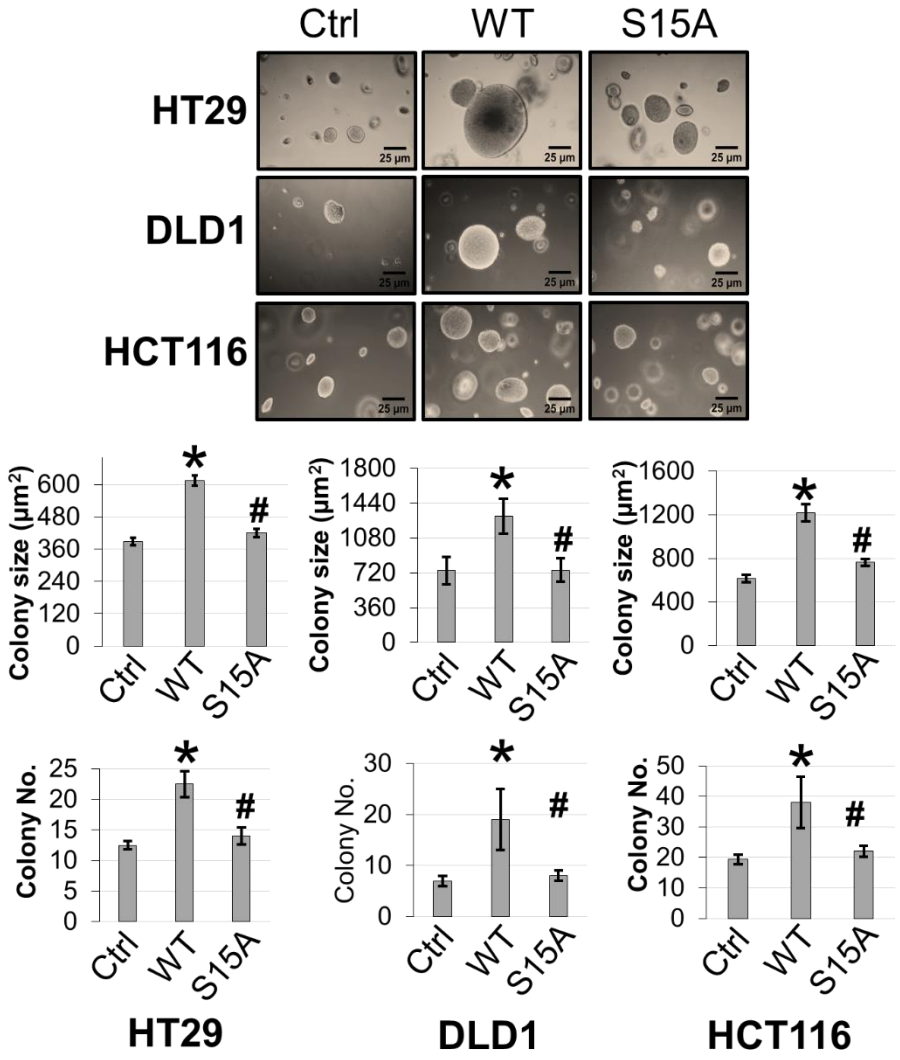


Figure 19: S15 phosphorylation mediates CRC anchorage-independent growth associated with PRMT5 overexpression. **Top panel:** Anchorage-independent growth assay with colon cancer cells overexpressing WT-PRMT5 (WT) or S15A-PRMT5 (S15A) mutant compared to vector control (Ctrl). Representative images of colonies for HT29, DLD1 and HCT116 are shown. **Bottom panel:** Quantification of the average colony size and number is shown below the corresponding cell type. The data represent the means ± standard deviation (S.D.) for three independent experiments. **p<0.05 vs. Ctrl group; #p<0.05 vs. WT group.*

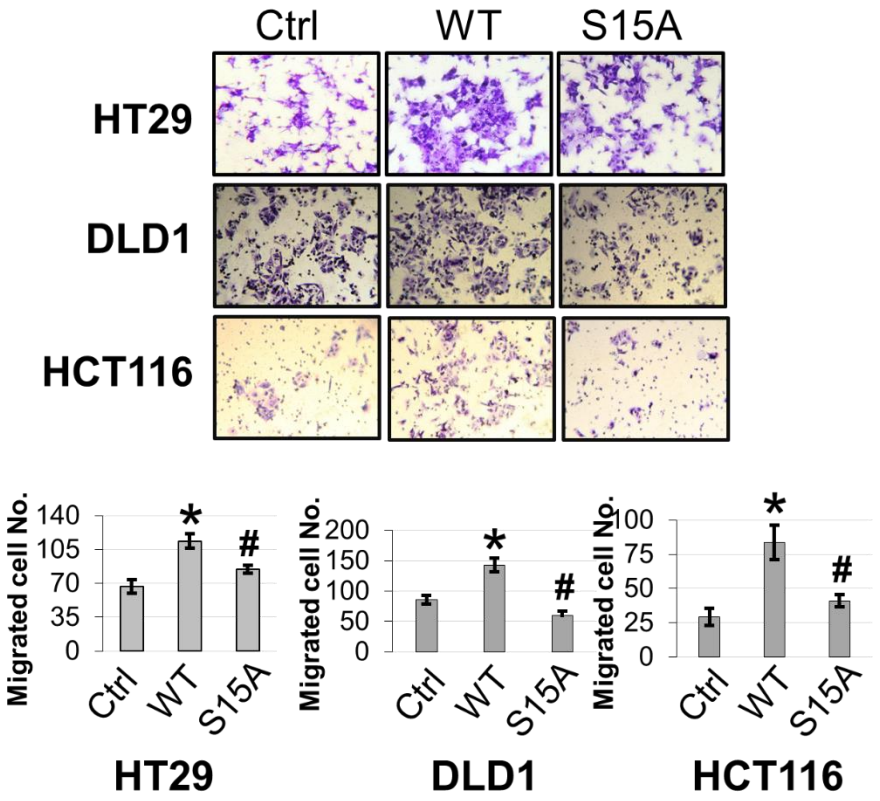


Figure 20: S15 phosphorylation mediates CRC cell migration associated with PRMT5 overexpression. **Top panel:** Boyden chamber transwell assay, showing migration of colon cancer cells overexpressing WT-PRMT5 (WT) or S15A-PRMT5 (S15A) mutant compared to vector control (Ctrl). Representative photos of crystal violet stained cells are shown with 20X magnification. **Bottom panel**: Quantification of the average number of migrated cells is shown. The data represent the means ± standard deviation (S.D.) for three independent experiments. **p<0.05 vs. Ctrl group; #p<0.05 vs. WT group.*

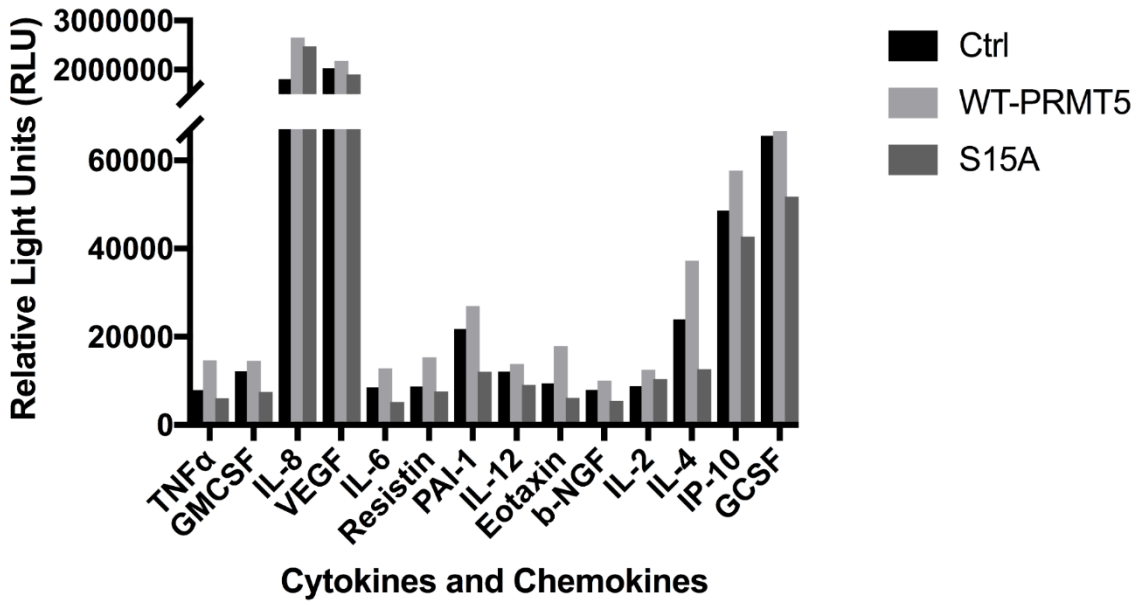


Figure 21: S15 phosphorylation is important for cytokine and chemokine secretion. Analysis of cytokine expression in conditioned media from WT-PRMT5- or S15A-PRMT5 -overexpressing HT29 cells using a Human Cytokine ELISA Array. Total of 32 Cytokines/Chemokines were tested.

4.3 Summary and Discussion

CRC is a leading cause of cancer-related mortality worldwide. One of the main challenges of treating CRC is the fact that is a highly heterogeneous disease, and besides standard chemotherapy and a few biologics, the availability of effective targeted treatment options for patients with advanced disease is quite limited. This indicates a clear unmet clinical need which continues to fuel interest for developing new approaches to prevent metastasis and inhibit tumor growth based on the molecular profile of CRC tumors, with the goal of improving prognosis for these patients. Aberrant activation of the transcription factor nuclear NF-κB is observed in 50-60% of CRC cases, and is increasingly recognized as a crucial etiological factor in many of the steps of cancer progression and chemotherapeutic resistance. Importantly, the ability of NF-κB to exert its pro-tumorigenic functions is often linked to its cooperativity with other cancer-associated proteins. Recently, PRMT5 has emerged as an important biomarker for CRC malignancy and increasing evidence argues that enhanced expression of PRMT5 drives cancer cell growth and survival through its methylation of substrates involved in oncogenic signaling. Importantly, our lab identified a critical interplay between PRMT5 and NF-κB with implications in cancer.

In **Chapter 3**, we provided strong evidence that overexpression of PRMT5 in CRC cells significantly augmented the activation of NF-κB through symmetric dimethylation of p65 in an S15 phosphorylation-dependent manner. We then hypothesized that this phosphorylation event could contribute to promoting cancer-associated characteristics accompanying overexpression of PRMT5 in part, by mediating activation of NF-κB. To this end, the work in this chapter demonstrated that when compared to wild-type PRMT5, the S15A-PRMT5 mutant significantly attenuated the growth, migratory and colony-forming abilities of CRC cells, thus establishing functional significance for this PTM. Although the list of PTMs that regulate PRMT5 has

grown over the last few years, insight into the biological relevance of these modifications is still significantly lacking. Furthermore, their relative importance in pathological contexts remains to be established and are governed by factors yet to be identified. In this respect, our findings have made a significant contribution to the field.

As mentioned above, the tumor-associated properties of PRMT5 are mediated by its ability to methylate a number of key target proteins, including NF-κB. These PRMT5-catalyzed modifications can coordinately trigger the necessary changes in gene expression programs that promote cancer malignancy. The majority of documented studies also show that the biomarker and therapeutic potential of PRMT5 in cancers such as CRC is based on its elevated levels in cancer tissues compared to normal or benign counterparts. In fact, the success of many targeted therapies is based on the identification of specific somatic mutations and/or upregulation of proteins that can be modulated as a means of selectively destroying cancer cells. These aberrations are often indicative of an evolved dependence on these oncogenic factors for sustained tumor growth. In the case of PRMT5 for instance, a recent comprehensive study on functional characterization of cancer cell dependencies revealed that loss of the metabolic enzyme methylthioadenosine phosphorylase (MTAP) conferred a selective dependence on PRMT5 (Kryukov *et al.*, 2016). However, one caveat to consider is that PRMT5 is also essential for normal development and plays a critical role in cellular functions related to differentiation, spermatogenesis, hematopoiesis and splicing, among others. Therefore, while it is justified to develop potent mechanisms of inhibiting PRMT5 in the treatment of CRC and other cancers, rigorous *in vitro* and *in vivo* testing should be done to rule out the potentially adverse effects that PRMT5 inhibition might have on other vital physiological processes. One way of addressing any undesirable deleterious toxicities associated with full inhibition of PRMT5 is to determine the optimal therapeutic range of dosing that will effectively destroy cancer cells with negligible effects on normal

cells. The use of nanoparticle-based targeted drug delivery represents another attractive strategy to accomplish selectivity. In the future, it may also be advantageous to identify possible combinatorial approaches that exploit synergistic vulnerabilities should these toxicities become clinically dose-limiting.

Based on the findings presented in this chapter, we propose that overexpression of PRMT5 promotes some aspects of a malignant phenotype in CRC cells at least in part through its ability to enhance activation of NF-κB in an S15 phospho-dependent manner. We acknowledge however, that PRMT5-mediated signaling is inherently complex and NF-κB constitutes only one node in the possible range of signaling networks that phospho-PRMT5 might be acting through. Indeed, other PRMT5 substrates have been implicated in CRC including several histones and signaling factors such as EGFR, p53 and E2F-1. Thus, we cannot discount the possibility that the effects we observed with PRMT5 overexpression may be due to its direct or indirect regulation of these pathways. Moreover, due to its pleiotropic role, it is reasonable to assume that the contribution of NF-κB to the cancer phenotypes studied here may be exerted through both PRMT5-dependent and independent mechanisms. Further studies to delineate these points are needed. The development of high-throughput approaches to determine global changes in the symmetric dimethylation status of PRMT5 target proteins in tandem with correlative transcriptome analyses will allow us to better define which PRMT5-driven pathways are active in a given tumor subtype. This will allow for a more rational design of therapies and identification of patient subsets that will likely benefit from targeting specific pathways. Finally, as new information about the cross-talk between PRMT5 and other tumor regulatory pathways in CRC becomes available, studies aimed at targeting multiple dependencies at once by evaluating the efficacy of combining existing FDA-approved drugs with PRMT5 inhibitors should be considered.

4.4 Concluding Remarks

In summary, the work described here has revealed an important regulatory mechanism by which PRMT5 exerts its tumor-promoting functions in CRC, via S15 phosphorylation. This lends further support to the exploitation of PRMT5 as an important therapeutic target. Moreover, with the advent of multi-agent treatment modalities, it is critical to understand the mechanisms that govern cross-talk between various tumor-promoting factors with the intent of determining whether these signaling networks confer certain susceptibilities to cancer cells. In this respect, our data has shed important light on the functional cooperativity between arginine methyltransferases and kinases that converge on the NF-κB pathway, uncovering another potential avenue of therapy for CRC which involves limiting the activity of the PRMT5/NF-κB axis using selective kinase inhibitors. This represents an underexplored yet promising generation of anti-cancer drugs.

CHAPTER 5: PKCι PHOSPHORYLATES PRMT5 AND REGULATES PRMT5-MEDIATED ACTIVATION OF NF-κB

5.1 Background and Rationale

Our collective findings from **Chapters 3** and **4** demonstrate the potential to pharmacologically block the phosphorylation of PRMT5 using selective kinase inhibitors as the basis to disrupt PRMT5/NF-κB cooperativity and impede CRC growth. Hence, the identification of the appropriate kinase mediating phosphorylation of PRMT5 at S15 is of paramount importance in this process. Using the human protein reference database (HPRD), we identified the S15 residue as having a high probability of being phosphorylated by PKC based on a compendium of phosphorylation motifs. Interestingly, the only PKC isozyme identified as a PRMT5-interacting partner from our mass spectrometry protein identification studies was PKC iota (PKCι), belonging the family of atypical PKCs. Furthermore, the motif containing the S15 site bore striking similarity to a highly consensus atypical PKC's substrate recognition sequence pattern previously identified by another group (C. Wang *et al.*, 2012). Collectively, this argues for PKCι as a promising candidate for mediating phosphorylation of PRMT5. As proof of principle, validation studies to test this notion are outlined in this chapter.

5.2 Results

5.2.1 Identification of PKCι as an interacting partner of PRMT5

To determine which kinase(s) phosphorylates PRMT5 at S15, we first utilized the Human Protein Reference Database (http://www.hprd.org/ PhosphoMotif_finder) to predict PKC and PKA as putative kinases related to S15 phosphorylation based on consensus sequence phospho-motifs **(Figure 22A)**. To narrow down the top candidate kinase, we then identified the isozyme PKCι as an interacting partner of PRMT5 based on our mass spectrometry studies and further confirmed this interaction using co-

immunoprecipitation studies in HEK293 and HT29 cells. As shown in **Figure 22B**, Flag-WT-PRMT5 co-immunoprecipitated with endogenous PKCι under basal and IL-1β-stimulating conditions, validating the formation of a complex between PRMT5 and PKCι.

5.2.2 Knockdown of PKCι correlates to decreased serine phosphorylation of PRMT5 and disruption of the PRMT5-p65 complex

Next, we wanted to confirm that PRMT5 serves as a substrate of PKCι, and hence, we used a pool of shRNA constructs to knockdown the expression of PKCι concurrent with Flag-tagged WT-PRMT5 (Flag-WT) overexpression in HEK293 cells (**Figure 23A**). These cells were then treated with IL-1β, followed by immunoprecipitation of Flag-WT to detect PRMT5 phosphoserine (pSER) levels. As shown in **Figure 23B,** induction of serine phosphorylation of PRMT5 was observed under IL-1β-stimulating conditions in the cells co-expressing Flag-WT and shscramble constructs. By contrast, the Flag-WT protein purified from shPKCι cells showed diminished IL-1β-induced phosphorylation, confirming that PKCι mediates phosphorylation of PRMT5 in a ligand-inducible manner. Our next logical step was to examine whether PKCι could in turn regulate NF-κB signaling through disruption of the PRMT5-p65 complex. Stable HEK293 cells described in **Figure 23A** were subjected to co-immunoprecipitation. As shown in **Figure 23C**, knockdown of PKCι correlated with a disruption of the IL-1β-inducible PRMT5-p65 complex formation, similar to that previously observed with the S15A mutant (**Figure 14**).

5.2.3 PKCι regulates PRMT5-mediated NF-κB activation through S15 phosphorylation

To further test whether PKCι could regulate NF-κB activity through phosphorylation of PRMT5 at S15, we conducted NF-κB luciferase assays using HEK293 cells overexpressing vector control, WT-PRMT5 or S15A-PRMT5 constructs

with or without depletion of PKCι (**Figure 23A**). As shown in **Figure 24A**, knockdown of PKCι correlated with significantly decreased IL-1β-inducible NF-κB activity in vector control and WT-PRMT5- overexpressing cells compared to their shscramble counterparts. Consistent with our earlier findings, overexpression of the S15A-PRMT5 mutant significantly attenuated the activation of NF-κB compared to WT-PRMT5. Interestingly however, no further decrease in NF-κB activity in the IL-1β-treated S15A-PRMT5 /shPKCι cells was observed, suggesting that blockade of PKCι was likely acting through the S15 phosphorylation site of PRMT5. We sought to corroborate these observations using a selective small molecule PKCι inhibitor CRT0066854. We observed a similar phenomenon in which CRT0066854 disrupted IL-1β-inducible WT-PRMT5-mediated activation of NF-κB luciferase activity whereas no further decrease in NF-κB activity was detected in S15A-PRMT5 cells treated with the inhibitor under IL-1β-stimulating conditions (**Figure 24B**).

5.2.4 PKCι is significantly upregulated in human colorectal adenomas

PKCι has emerged as a bonafide oncogene in several types of cancers. Hence, based on the above findings, deregulation of PKCι in the context of CRC could be one potential mechanism that leads to aberrant phosphorylation of PRMT5 and in turn, downstream activation of NF-κB. Using an online TCGA-based database we initially assessed the relative transcript levels of PKCι in CRC tissues and found that in fact, it was highly upregulated in Stages I-IV compared to normal patient samples (**Figure 25**), indicating that PKCι may potentially have biomarker significance or be an actionable target in CRC (http://ualcan.path.uab.edu)(Chandrashekar *et al.*, 2017). We have outlined several validation experiments in the future directions section to address these latter points (**Chapter 6**).

A

Position	Sequence	Motif	Kinase
13 - 15	RVS	RXpS	PKA
13 - 15	RVS	[R/K]X[pS/pT]	PKA, PKC

B

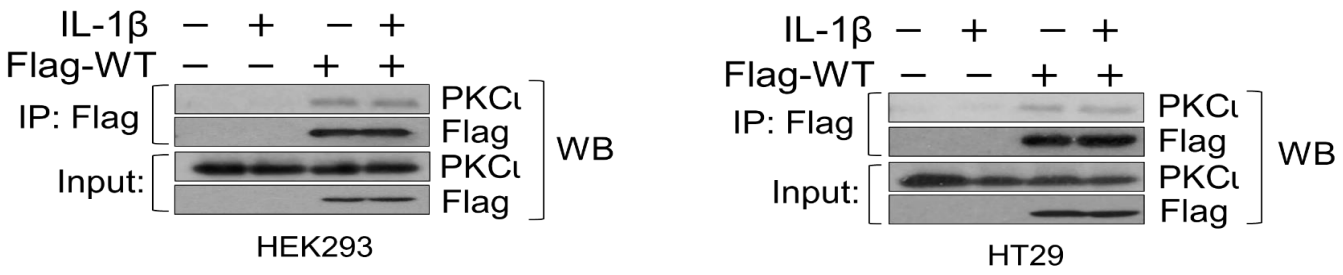


Figure 22: PKCι forms a complex with PRMT5. **A.** Human Protein Reference Database predicted PKC and PKA phospho-motif in position 13-15 of PRMT5. **B**. Co-immunoprecipitation (IP) experiments, HEK293 and HT29 cells were treated or left untreated with IL-1β (10ng/mL) for 1 h, Flag-WT-PRMT5 (Flag-WT) was immunoprecipitated with anti-Flag beads. Samples were then subjected to western blot analysis (WB) and probed with the indicated antibodies. Inputs were probed with anti- PKCι and anti-Flag antibodies.

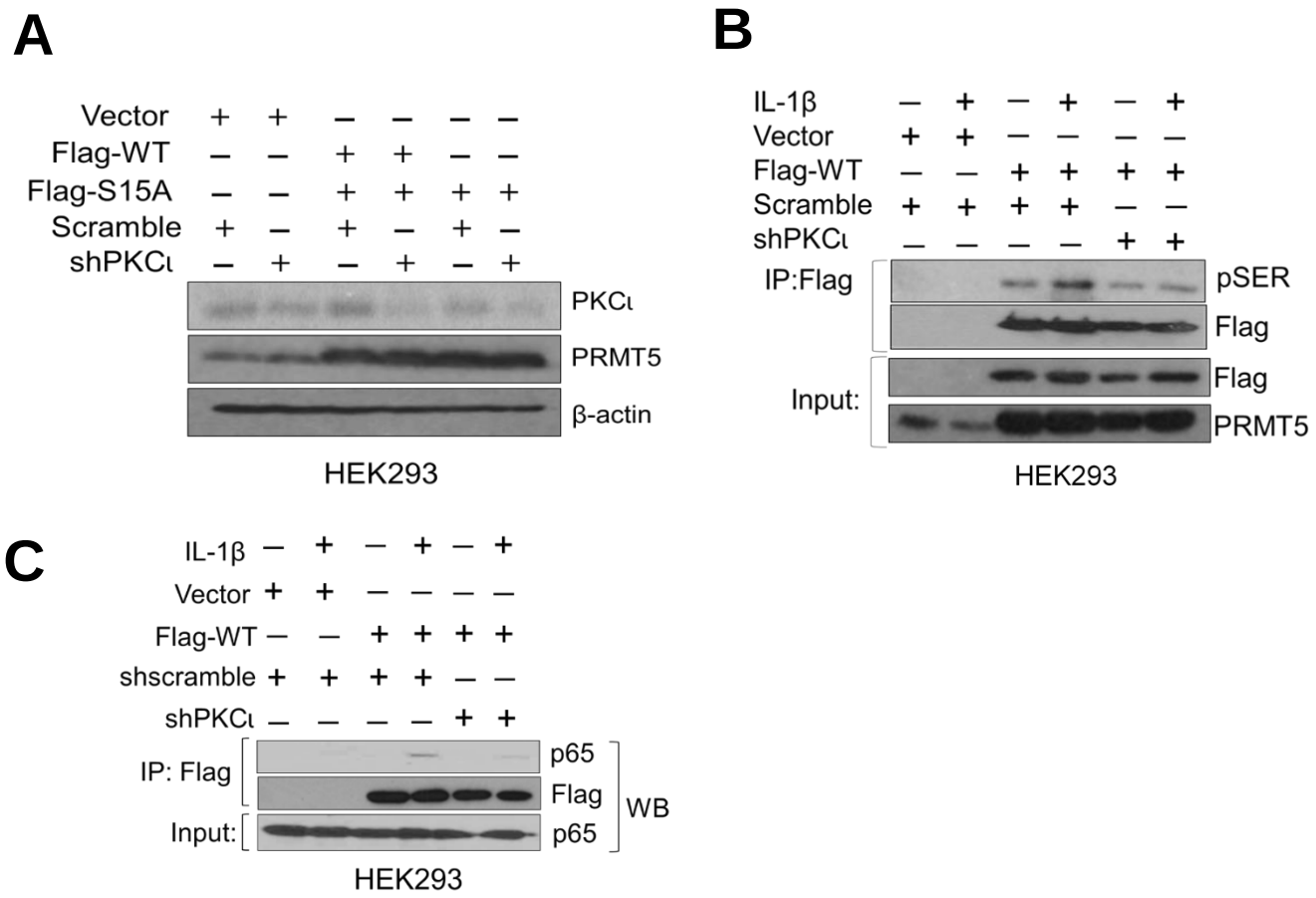
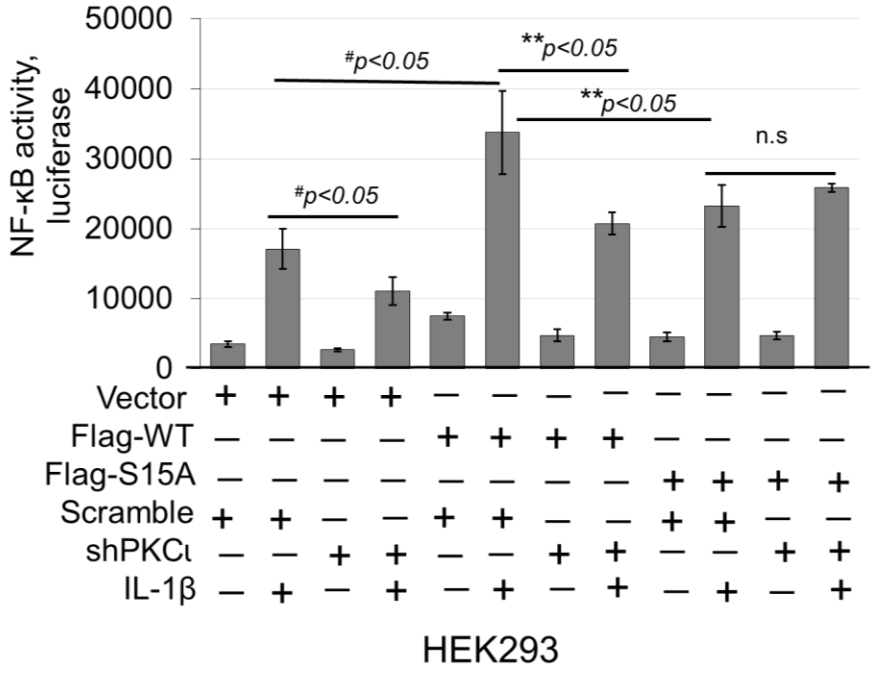
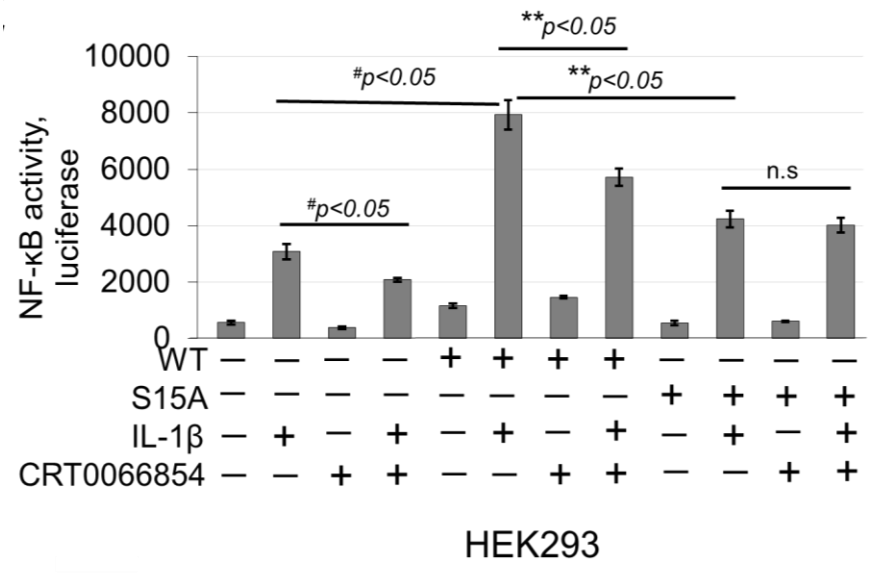


Figure 23: PKCι phosphorylates PRMT5 and regulates formation of PRMT5-p65 complex. **A.** Establishment of vector control, Flag-WT-PRMT5 or Flag-S15A-PRMT5- overexpressing HEK293 stable cells with concurrent expression of either shscramble or shPKCι constructs. Western blot image, probed with anti-PKCι, PRMT5, or β-actin respectively. **B**. Detection of PKCι-mediated serine phosphorylation of PRMT5 using co-immunoprecipitation and western blot analyses. Either HEK293 cells with vector control and shscramble, Flag-WT and shscramble or Flag-WT and shPKCι were treated with IL-1β or left untreated for 1 h (10 ng/mL). Samples were collected and Flag-WT was further immunoprecipitated with anti-FLAG beads and subjected to western analysis using an anti-phospho-serine motif antibody (pSER). The inputs were probed with anti-PRMT5 or Flag antibody. **C.** Co-immunoprecipitation (IP) experiment, HEK293 cells were treated or left untreated with 10 ng/ml of IL-1β for 1 h, WT-PRMT5 (Flag-WT) or S15A (Flag-S15A) was immunoprecipitated with anti-FLAG beads. Samples were then subjected to western blot analysis and probed with anti-p65 antibody. Inputs were probed with anti-p65 and anti-Flag antibodies.

A


NF-κB activity, luciferase
50000
40000
30000
20000
10000
0
#p<0.05
**p<0.05
**p<0.05
#p<0.05
n.s
Vector + + + + — — — — — — — —
Flag-WT — — — — + + + + — — — —
Flag-S15A — — — — — — — — + + + +
Scramble + + — — + + — — + + — —
shPKCι — — + + — — + + — — + +
IL-1β — + — + — + — + — + — +
HEK293

B


NF-κB activity, luciferase
10000
8000
6000
4000
2000
0
**p<0.05
#p<0.05
**p<0.05
#p<0.05
n.s
WT — — — — + + + + — — — —
S15A — — — — — — — — + + + +
IL-1β — + — + — + — + — + — +
CRT0066854 — — + + — — + + — — + +
HEK293

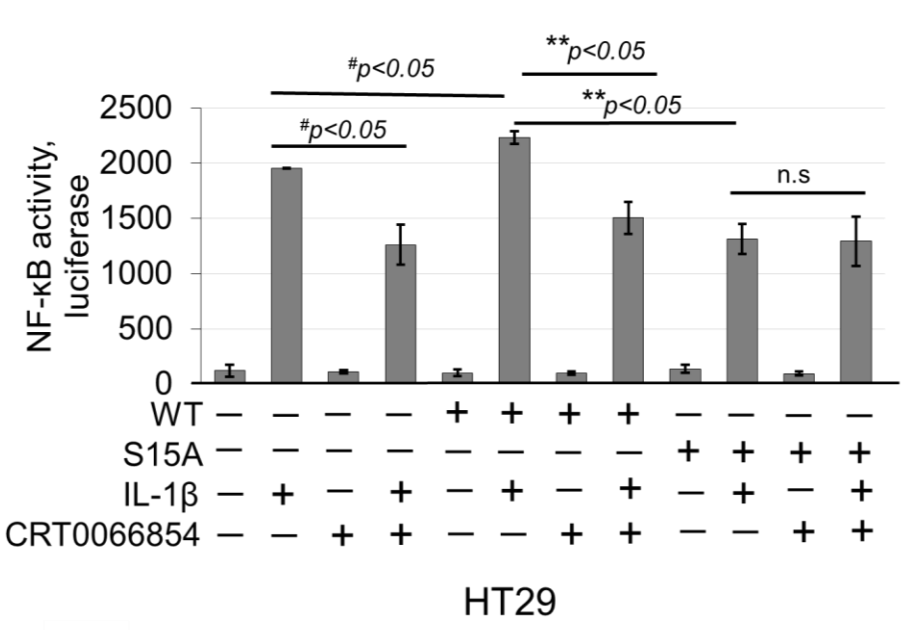
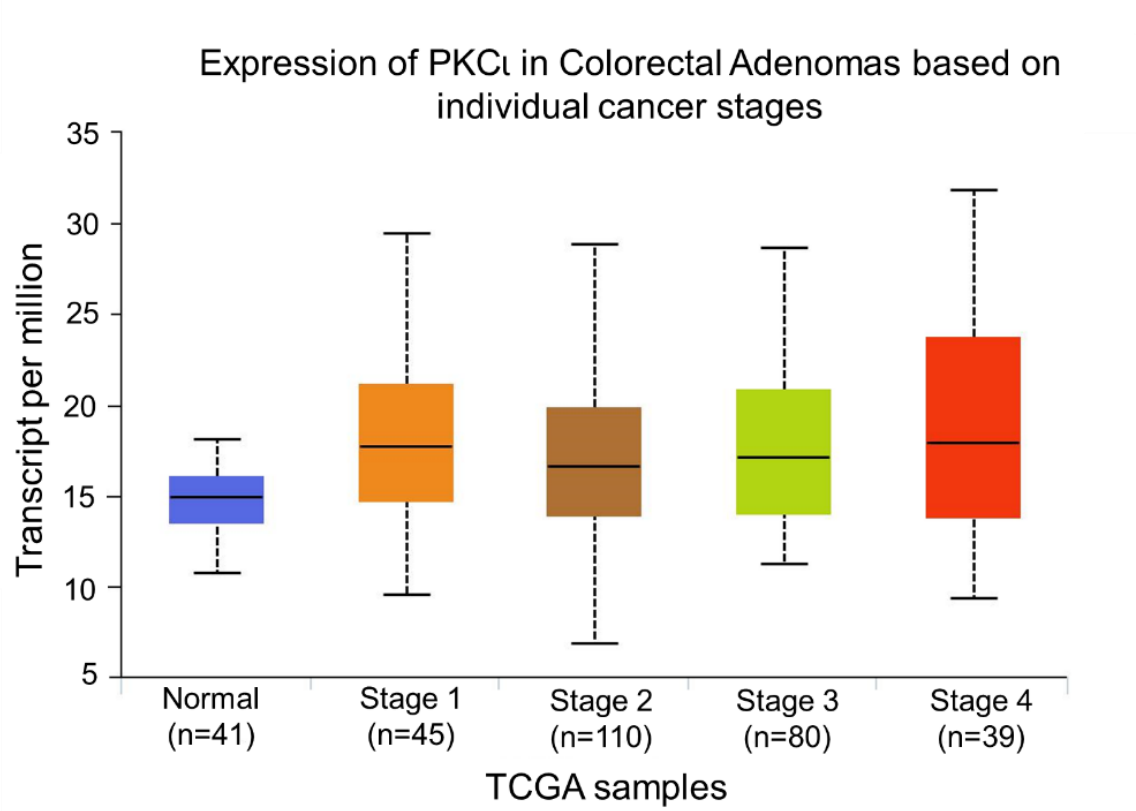


Figure 24: PKCι regulates PRMT5-mediated NF-κB activation through S15 phosphorylation. NF-κB activity was determined by luciferase assay, in established cells shown above (**Figure 22A)**. **A.** NF-κB activity luciferase assay in vector control or cells overexpressing WT-PRMT5 (WT) or S15A-PRMT5 with or without knockdown of PKCι in the presence or absence of IL-1β stimulation. **B.** NF-κB activity luciferase assay in control or cells overexpressing WT-PRMT5 (WT) or S15A-PRMT5 treated with or without PKCι small molecule inhibitor, CRT0066854 in the presence or absence of IL-1β stimulation in HEK293 and **C**. HT29 cells. The data represent the mean ± SD from three independent experiments. *$^{\#}$p<0.05 vs. Ctrl+IL-1 β group; **p<0.05 vs. WT+IL-1β group; n.s: not significant.*



Comparison	Statistical significance
Normal-vs-Stage1	1.997810E-04
Normal-vs-Stage2	6.11209999945572E-07
Normal-vs-Stage3	2.39080000019598E-07
Normal-vs-Stage4	2.034100E-04

Figure 25: PKCι is significantly upregulated in colorectal cancer patient samples. **Upper panel,** Boxplot showing relative transcript levels of PKCι in normal and colorectal adenoma human samples based on individual cancer stages. **Bottom panel,** Log-rank test was used to indicate statistical significance between normal and each cancer stage (http://ualcan.path.uab.edu).

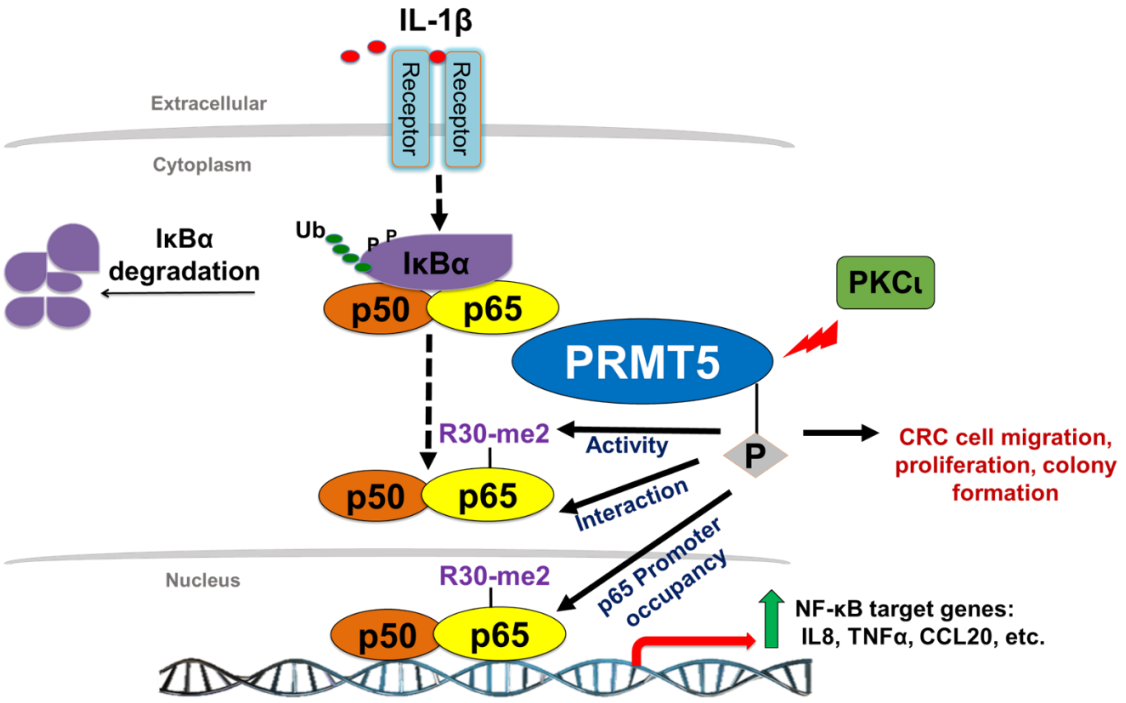


Figure 26: Hypothetical model. IL-1β stimulation activates the NF-κB pathway and induces PKCι-mediated phosphorylation of PRMT5. Phosphorylation of PRMT5 at S15 elicits formation of the PRMT5-p65 complex and regulates PRMT5-dependent proximal promoter occupancy of p65 at target genes. Collectively, these constitute pivotal mechanisms by which PRMT5 can fine-tune NF-κB activation and target gene expression. Furthermore, S15 phosphorylation mediates IL-1β-induced PRMT5 activity and in turn the R30 methylation of p65. These signal-induced effects potentially serve to facilitate the pro-tumor functions exerted by PRMT5.

5.3 Summary and Discussion

Protein kinases belong to one of the largest families of enzymes and are known to play key roles in nearly all cellular functions including signal transduction, cell cycle regulation, cell division, apoptosis and cell differentiation, among others. Of the 518 plus protein kinase genes in the human genome, approximately 372 belong to the class of serine/threonine kinases (STKs), some of which are receptors or dual specificity protein kinases (tyrosine and serine/threonine) (Roskoski, 2015). Using a combined approach of co-immunoprecipitation, mass spectrometry protein identification and PhosphoMotif Finder we identified the STK PKCι as a potential candidate kinase that catalyzes the phosphorylation of PRMT5 at S15. PKCs are widely known as important regulators of signal transduction cascades. These isozymes are divided in 3 major subcategories based on the signals that activate them such as diacylglycerol (DAG) or calcium ions (Ca^{2+}): conventional PKCs (α, βI, βII, and γ) require Ca^{2+}, DAG, and a phospholipid such as phosphatidylserine for activation whereas novel PKCs (δ, ε, η, and θ) need DAG, but not Ca^{2+} for activation. PKCι belongs to the third class, namely atypical PKCs (Mζ , ι / λ), which require neither Ca^{2+} nor DAG for activation. Like many kinase families, PKCs share some key conserved structural motifs such as a catalytic domain, activation loop and ATP binding domain (Newton, 1995).

Here, we demonstrated that not only does PRMT5 complex with PKCι but shRNA-mediated depletion of this kinase corresponded with less IL-1β-inducible serine 15 phosphorylation of PRMT5. Furthermore, using luciferase assays, we showed that knockdown or pharmacological inhibition of PKCι could impair transactivation of p65 in a phosphoPRMT5-dependent fashion, indicating that PKCι may be acting upstream of both PRMT5 and NF-κB. Thus, not only have we established a novel link between PKCι, phosphorylation of PRMT5 at S15 and NF-κB activation, but this discovery may have implications in cancer. PKCι has been characterized as an oncogene and is implicated in

the initiation and progression of colitis-associated colon cancer via regulation of epithelial cell integrity and polarity (Calcagno *et al.*, 2011). Moreover, mutations and genetic alterations of PKCι are often detected across various cancer types including ovarian and breast cancer, making it a highly attractive therapeutic target (Nanos-Webb *et al.*, 2016; Paul *et al.*, 2014). PKCι upregulation has also been shown to activate pathways involved in certain hallmarks of cancer including proliferation, anchorage-independent growth and epithelial to mesenchymal transition (EMT) (Gunaratne *et al.*, 2013).

Interestingly, a few accounts linking PKCι and NF-κB have been documented and enhanced expression of PKCι was shown to protect cancer cells from apoptosis by activating NF-κB, although the exact underlying mechanisms are still unclear (Parker *et al.*, 2014). For instance, in MDA-MB-468 breast cancer cells, cytokine-mediated activation of NF-κB signaling was shown to promote the association of PKCι with IKKβ resulting in nuclear translocation of p65 (Paul *et al.*, 2014). Similarly, in Th2 cells, PKCι, whose levels were increased during Th2 differentiation, was shown to activate NF-κB through its complex with IKKβ (Martin *et al.*, 2005; Yang *et al.*, 2009). Two separate studies defined a novel pathway for the activation of NF-κB involving PKCι, p62 and IKKβ in which expression of a dominant-negative mutant PKCι impaired RIP-stimulated NF-κB by disrupting the association between RIP, p62 and IKKβ (Diaz-Meco *et al.*, 2012). Collectively, these studies suggest that the role of PKCι in activation of NF-κB is perhaps mediated by other cellular signaling factors. Of great functional relevance in this regard is our finding that disruption of PKCι-mediated phosphorylation of PRMT5 could significantly impair IL-1β-stimulated activation of NF-κB. Moreover, in cells expressing the S15A-PRMT5 mutant, inhibition of PKCι had negligible effects, strongly suggesting that activation of NF-κB by PRMT5 was mediated at least in part by this phosphorylation event.

Our data presents a complex picture which involves a signaling cascade of interactions between these important molecules. However, signal transduction events can be inherently complicated, and our data raises several questions that remain to be experimentally tested. First, we acknowledge that kinases may phosphorylate more than one site on their substrates and thus we cannot exclude the possibility that PKCι may phosphorylate other sites on PRMT5 yet to be identified. Furthermore, there may be cross talk between S15 phosphorylation and other PTMs on PRMT5 which may concertedly play a role in its activation of NF-κB. These are possibilities we would like to ultimately explore. Second, we have shown that PRMT5 complexes with and is a substrate of PKCι, however, the intricacies of these associations and their relation to p65 require further investigation. For instance, our co-immunoprecipitation studies showed that the complex between PRMT5 and p65 can be enhanced by IL-1β whereas formation of the PRMT5/PKCι complex remained consistent before and after IL-1β stimulation. Interestingly however, knockdown of PKCι diminished the IL-1β-induced phosphorylation of PRMT5, suggesting that other factors are likely at play. We propose that one possibility is that instead of modulating the complex between PRMT5 and PKCι, IL-1β may trigger a change in the activation state of PKCι. In turn, activated PKCι promotes phosphorylation of PRMT5, facilitating a stronger complex between PRMT5 and p65. To test these notions, we will conduct future studies to determine whether IL-1β induces phosphorylation of PKCι at sites known to be involved in its activation. This is in keeping with a previous study described by the LaVallie group in which they showed that IL-1β treatment of chondrocytes in culture resulted in rapidly increased phosphorylation of another atypical PKC isozyme, namely PKCζ (LaVallie *et al.*, 2006).

Next, using a combination of co-immunoprecipitation and western blot approaches, we will probe whether knockdown of PKCι disrupts the PRMT5/p65 complex as well as the R30 methylation status of p65. In terms of understanding the

spatiotemporal dynamics of this signaling cascade, we will also conduct co-immunoprecipitation studies within different cell fractions to determine where this critical PKCι/PRMT5/NF-κB complex is formed. Finally, considering the afore-mentioned reports, it will be interesting to investigate whether in our system a similar association between PKCι and other components of the NF-κB pathway such as IKKβ exists under IL-1β-stimulating conditions. Taken together, these experiments will provide a more detailed and mechanistic depiction of these complex signaling relationships.

5.4 Concluding Remarks

We have identified PRMT5 as a novel substrate of the oncogene PKCι. Moreover, we demonstrate that PKCι serves as an important signaling component of IL-1β-induced and PRMT5-mediated activation of NF-κB, an effect blocked by knockdown or selective inhibition of PKCι. Collectively, these studies suggest that the cancer-associated role of PRMT5 may in part be linked to its functional interaction with certain growth promoting proteins, including PKCι and NF-κB. We posit that this in turn influences its biological activities in such a way to favor hallmark characteristics of cancer including invasiveness, cell growth and survival. To this end, further experimental studies are needed to validate these latter points. Specifically, we will determine whether a corresponding reduction in the migratory, proliferative and anchorage-independent growth of CRC cells is observed upon knockdown or inhibition of PKCι, similar to the effect of overexpressing the S15A mutant of PRMT5 (**Chapter 4**). Likewise, we would expect an overlap in the subset of NF-κB target genes downregulated by PKCι depletion and S15A-PRMT5 overexpression. In conclusion, our data provides the basis for exploiting the therapeutic potential of disrupting the PKCι/PRMT5/NF-κB signaling axis in CRC. Importantly, these applications could potentially be generalized to other tumor types.

CHAPTER 6: CONCLUDING DISCUSSION

6.1 Challenges in the clinical management of CRC

CRC remains one of the leading causes of cancer-related deaths worldwide. Although significant progress has been made in its clinical management, several challenges hinder the overall effectiveness of current treatments. For instance, surgery remains the most effective treatment for early-stage CRC, however, a significant fraction of patients present with advanced or metastatic disease at the time of diagnosis and of these, only 10-25% are resectable. Hence, aggressive combination chemotherapy has become the mainstay for disease management. Currently, FOLFIRI and FOLFOX in combination with either cetuximab (anti-EGFR) or bevacizumab (anti-VEGF) are used as first-line treatments. Unfortunately, chemotherapeutic resistance remains a major impediment and hence, many patients with advanced CRC may not experience considerable clinical benefit from these treatment combinations. Moreover, many patients experience toxic reactions such as nausea, vomiting, diarrhea, neuropathy, hair loss and increased risk of infections, resulting in treatment withdrawal or suboptimal dosing which can severely hamper treatment efficacy.

Over the past decade, research efforts have shifted towards identifying more reliable and validated therapeutic targets that are likely to have the greatest clinical outcome while minimizing adverse effects. To this end, molecular profiling and mutation analyses have become critical to CRC treatment individualization, facilitated by rapid advances in genomic, transcriptomic and proteomic technologies (Tran *et al.*, 2015). Discoveries from these approaches have fueled the development of novel drug targets and new treatment strategies. However, translation of these findings into routine clinical practice has been limited due to factors such as poor reproducibility in preclinical animal experiments and an overall lack of models that accurately predict clinical benefit. Going

forward, it will be imperative to establish more robust preclinical models that monitor drug responsiveness signatures in concert with molecular categorization of patient tissues. Additionally, a better understanding of the tumor microenvironment including the interaction between cancer cells and the host immune system should be considered when developing new therapies. These approaches will become essential to guiding clinicians to make more informed treatment decisions, including optimal sequential or combinatorial use of selected agents.

6.2 Key points for consideration

Phosphorylation-site analyses can provide important and definitive information on functional relationships between signaling proteins. In some phosphoregulation analyses, mutation of phosphorylation residues to generate “phosphomimetics” has become common in an attempt to study the constitutively phosphorylated state of the protein. In this approach, serine residues are typically mutated to aspartic (S-to-D) or glutamic acid (S-to-E). There are several limitations to this approach. First, if the phosphorylation site serves as a recognition signal for an adaptor protein (e.g., 14-3-3), phosphomimetic mutants will not bind to the adapter protein. One of the primary functions of adaptor proteins is to sequester and position client molecules to interact with their binding partners. Hence, one can speculate that in our case, a phosphomimetic mutant of PRMT5 may impair its ability to bind to a potential adaptor protein and in turn disrupt its complex with p65. A second limitation is the discrepancy in the negative charge introduced by the aspartate or glutamate residues and the phosphorylated serine at physiological pH which tend to be quite different.

Moreover, compared to the negatively charged aspartate or glutamate residues, the relative size of the ionic shell produced by a phosphate group creates a very different chemical environment and thus, phosphomimetic mutations often fail to recapitulate the

changes to a protein conferred by phosphorylation. Consequently, although there are examples in which phosphomimetic substitutions have been informative, the general effects of these mutations are often not easily interpretable.

6.3 Future Directions

Based on the exciting work presented in this thesis as summarized in **Figure 26**, we can infer that phosphorylation of PRMT5 at S15 is critical to the regulation of the PRMT5/NF-κB signaling axis (**Chapter 3**) and mediates some of the hallmark cancer-associated phenotypes exerted by PRMT5 in CRC (**Chapter 4**). Moreover, from our kinase identification studies in **Chapter 5**, PKCι emerges as a critical upstream regulator of both PRMT5 and the NF-κB pathway. Altogether, these findings suggest that pharmacological disruption of this axis using PKCι inhibitors could serve as the basis for new therapeutics that impede CRC progression. This opens up several avenues of exciting future studies. Currently, there are only a limited number of pan inhibitors on the market against PKCs but to our knowledge, few to none with high selectivity for PKCι. Hence, one of our immediate goals is to identify candidate PKCι inhibitors with the possibility of generating more efficacious derivatives of the most promising compounds. Initially, we will utilize a combined approach that includes structural docking analyses and *in vitro* screens of compound libraries to identify small molecule inhibitors with high selectivity for PKCι over other PKC isozymes. Next, we will test the efficacy of top compounds in impeding CRC growth using complementary cell-based and animal-based models of disease such as 3-D cultures, xenograft and orthotopic mouse models. Finally, to establish whether our lead compound selectively disrupts the PRMT5/NF-κB axis in our *in vivo* models, we can probe the mouse tissue to see if we observe a correlative decrease in the levels of S15 phosphorylated PRMT5, p65 dimethylation accompanied by a resultant decreased expression of relevant NF-κB target genes.

Another important area of research to pursue involves elucidating the structural intricacies of the PRMT5/p65/PKCι complex. First, we will undertake detailed co-immunoprecipitation-based mapping of the interaction domains between the three proteins to determine if they directly bind and if so, which critical residues are involved in their interaction. This data will be valuable for informing molecular dynamics simulation (MDS) studies for determining potential binding "hotspots", which are defined as a cluster of residues that makes a major contribution to the binding free energy (Brinda *et al.*, 2005). Intriguingly, previous reports have shown that phosphorylation sites are frequently located on binding interfaces which can then serve as important hotspots that modulate the strength of protein-protein interactions by causing significant changes in binding energy (Nishi *et al.*, 2011). Hence, using the MDS platform, it would be interesting to determine if phosphorylation of PRMT5 at S15 serves as one such hotspot and investigate how it might mediate the protein-protein interactions previously characterized by us. These experiments may necessitate co-crystallization of PRMT5 with p65 and PKCι.

Furthermore, we have shown for the first time that S15 phosphorylation constitutes an important positive regulator of the IL-1β-inducible methyltransferase activity of PRMT5. Interestingly, our findings stand in contrast to a previous study that identified tyrosine phosphorylation (Tyr297, Tyr304, Tyr307) of PRMT5 by mutant Jak2 as playing an inhibitory role on PRMT5's activity. It is important to note that these studies were carried out under very different cellular contexts. Whether these tyrosine phosphorylation events are induced by stimuli other than IL-1β is currently unknown since we did not detect these modifications in our mass spectrometry analyses carried out using IL-1β-treated cells. Hence, the previous functional implications of these particular tyrosine PTMs may not necessarily be relevant in our system. However, it would be interesting to compare the differential effects conferred by S15 phosphorylation

versus tyrosine phosphorylation purely at the structural level. This may provide deeper molecular insight into the unique function of distinct PTMs in regulating PRMT5 enzymatic activity through potentially divergent conformational changes.

Finally, we acknowledge that CRC like other cancers, is a highly heterogeneous disease and the “curative” potential of a single targeted therapeutic agent would be an unrealistic expectation. Thus, continued efforts to determine the optimal combinations of compounds and/or drugs for the treatment of CRC patients are needed. Along these lines, we will consider the combined efficacy of our lead PKCι inhibitor with other mainstream chemotherapeutic regimens and/or targeted agents. Indeed, some of these approved agents (e.g., anti-EGFR drugs) target other pathways in which PRMT5 has been implicated. However, we are yet to determine if the role of S15 phosphorylation extends beyond PRMT5-mediated activation of NF-κB or if methylation of other PRMT5 substrates plays a role in CRC progression. If these pathways or substrates are proven to be important in our models, more detailed studies can be conducted to determine the correct timing and context of using these multi-targeted combinatorial treatment approaches.

APPENDICES

Appendix A. Permissions

Figures 2, 3 and 4 were taken from the previously published work:
Hartley AV, Wei H, Prabhu L, Martin M, and Lu T. *NF-κB: Its role in colorectal cancer.* In: Transcriptional factors: their role in gastrointestinal malignancies. 2018. Ganji Purnachandra Nagaraju & P. Veera Bramha Chari (Ed.). Springer Nature Singapore; ISBN 978-981-10-6727-3.

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Requestor Location	Ms. Antja-Voy Hartley 1101 E 17th ST INDIANAPOLIS, IN 46202 United States Attn: Ms. Antja-Voy Hartley
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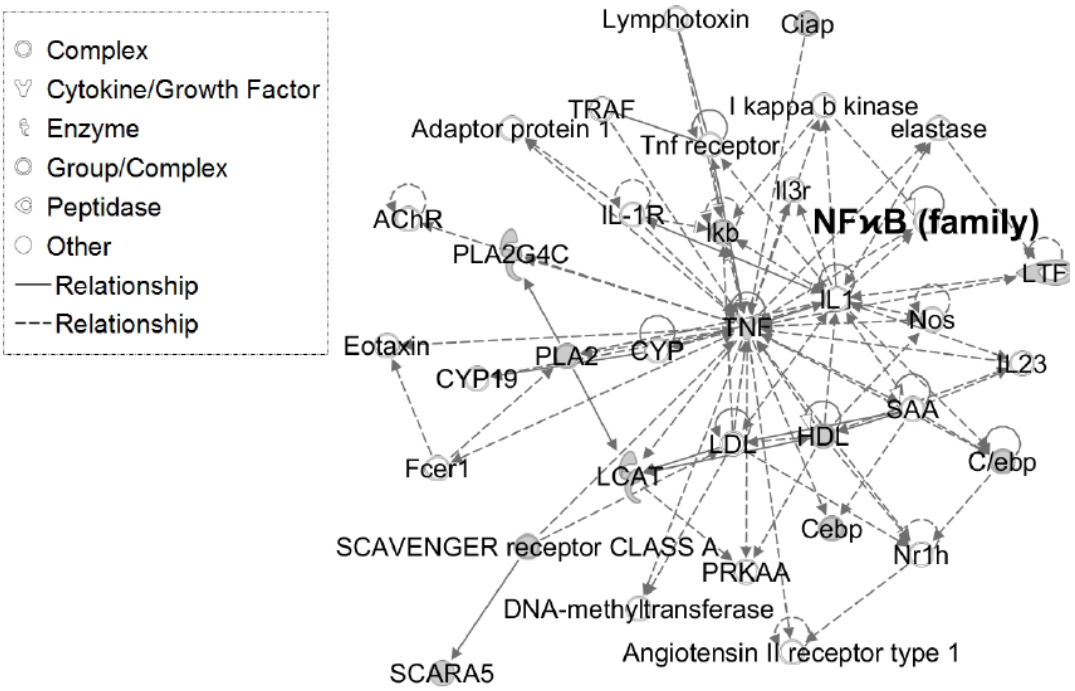
Appendix B. List of qPCR primers

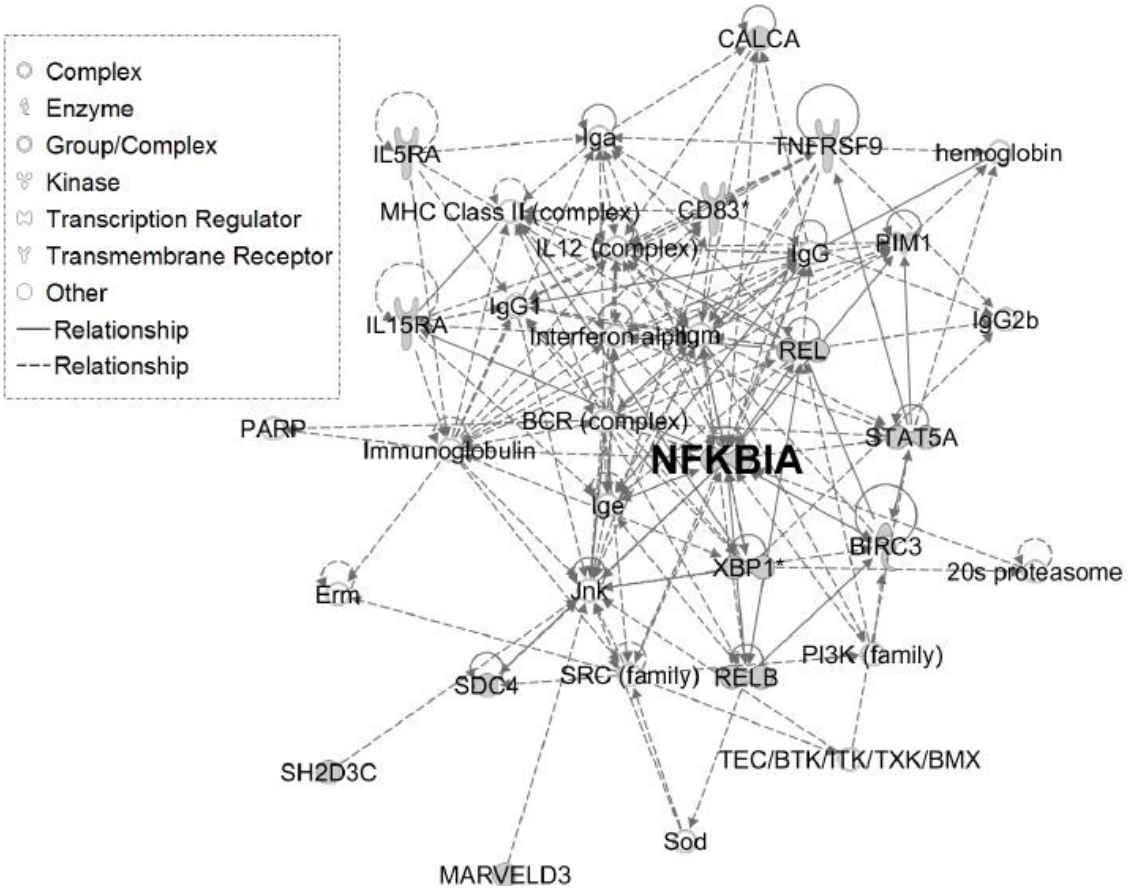
Gene Name	Primer	Lab Record	Primer Sequence Used
GAPDH	Forward	F326	5'-CCATCACCATCTTCCAGGAGCG-3'
	Reverse	R468	5'-AGAGATGATGACCCTTTTGGC-3'
IL8	Forward	F139	5'-TCCTGATTTCTGCAGCTCTGT-3'
	Reverse	R245	5'-AAATTTGGGGTGGAAAGGTT-3'
CCL20	Forward	F112	5'-GTGCTGCTACTCCACCTCTG-3'
	Reverse	R219	5'-CGTGTGAAGCCCACAATAAA-3'

Appendix C. List of primers used for site mutagenesis of PRMT5

Primer Name	Primer	Primer Sequence Used for Mutagenesis
PRMT5-S15A-PRMT5	Forward	5'-GGAGCCGCGTGGCCAGCGGGAGG-3'
	Reverse	5'-CCTCCCGCTGGCCACGCGGCTCC-3'
PRMT5-E444D	Forward	5'-CACTCAGGCGACAAATCATTGTCAGCAAATGAGCCC-3'
	Reverse	5'-GGGCTCATTTGCTGACAATGATTTGTCGCCTGAGTG-3'

Appendix D. Ingenuity Pathway Analysis Networks.
IPA representative networks showing genes regulated by S15A-PRMT5 with NF-κB as one of the critical nodes in these networks.




Complex
Enzyme
Group/Complex
Kinase
Transcription Regulator
Transmembrane Receptor
Other
Relationship
Relationship
CALCA
IL5RA
Iga
TNFRSF9
hemoglobin
MHC Class II (complex)
CD83*
IL12 (complex)
IgG
PIM1
IgG1
IL15RA
Interferon alpha/gamma
REL
IgG2b
PARP
BCR (complex)
Immunoglobulin
NFKBIA
STAT5A
Ige
BIRC3
XBP1*
20s proteasome
Erm
Jnk
PI3K (family)
SDC4
SRC (family)
RELB
SH2D3C
TEC/BTK/ITK/TXK/BMX
Sod
MARVELD3

Appendix E. Full list of genes downregulated by the S15A-PRMT5 mutation. Fold change is represented as S15A-PRMT5 +IL-1β/WT+IL-1β ≤ 0.5

ACCESSION NO.	TargetID	S15A-PRMT5 +IL-1β/WT+IL-1β
NM_001115.1	ADCY8	0.45
NM_014391.2	ANKRD1	0.38
NM_020373.2	ANO2	0.15
XR_041624.1	C10ORF85	0.36
XR_041485.1	C13ORF29	0.27
XM_001726191.1	C19ORF29OS	0.00
NM_178342.2	C3ORF35	0.50
NM_018452.3	C6ORF35	0.32
NM_145028.3	C6ORF81	0.16
NM_152786.1	C9ORF43	0.33
NM_001742.2	CALCR	0.24
NM_175931.1	CBFA2T3	0.28
NM_138414.1	CCDC101	0.41
NM_015603.1	CCDC9	0.48
NM_004591.1	CCL20	0.37
NM_207007.2	CCL4L2	0.47
NM_145057.2	CDC42EP5	0.41
NM_000735.2	CGA	0.33
NM_000737.2	CGB	0.45
NM_024111.2	CHAC1	0.00

NM_015557.1	CHD5	0.27
NM_024944.2	CHODL	0.47
NM_138429.1	CLDN15	0.49
NM_080645.2	COL12A1	0.22
NM_002089.3	CXCL2	0.29
NM_016229.3	CYB5R2	0.36
NM_000779.2	CYP4B1	0.31
NR_024064.1	DAD1L	0.39
NM_006557.4	DMRT2	0.28
NR_024595.1	DNM1P35	0.06
NM_133637.1	DQX1	0.37
NM_004428.2	EFNA1	0.48
NM_007036.2	ESM1	0.22
NM_153606.2	FAM71A	0.50
NM_001012426.1	FOXP4	0.06
NM_000148.2	FUT1	0.35
NM_002068.1	GNA15	0.40
XM_935238.1	GOLGA8F	0.45
BX109627	HS.130639	0.46
AI801879	HS.144030	0.14
BU633914	HS.25555	0.27
AK026734	HS.287720	0.34
AF339771	HS.344872	0.19
CD695721	HS.538157	0.00

AI253067	HS.541845	0.20
U10515	HS.544238	0.46
AI628074	HS.545238	0.13
NM_130770.2	HTR3C	0.28
NM_172200.1	IL15RA	0.17
NM_001012633.1	IL32	0.49
NM_000564.2	IL5RA	0.50
NM_000584.2	IL8	0.48
NM_002195.1	INSL4	0.30
XM_934728.1	KIAA0565	0.09
NM_138343.2	KLC4	0.02
NM_002774.3	KLK6	0.34
NM_004139.2	LBP	0.29
NM_001010939.1	LIPJ	0.18
NM_033029.2	LMLN	0.15
XM_001724965.1	LOC100129268	0.13
XM_001716704.1	LOC100130288	0.15
XM_001718675.1	LOC100130705	0.50
XM_001714361.1	LOC100131999	0.18
XR_038987.1	LOC100132496	0.37
XM_001724630.1	LOC100132716	0.05
XM_001726146.1	LOC100132839	0.06
XM_001721522.1	LOC100134009	0.18
XM_001720931.1	LOC100134041	0.14

XM_001715304.1	LOC100134081	0.00
XM_001721704.1	LOC100134170	0.19
XM_001714134.1	LOC100134499	0.00
NM_178514.3	LOC283487	0.26
XM_944838.2	LOC285733	0.27
XM_939888.1	LOC339742	0.49
XM_370865.4	LOC388122	0.23
XM_374766.2	LOC399715	0.11
XM_495854.3	LOC440013	0.10
XM_001717499.1	LOC642076	0.04
NR_024495.1	LOC642826	0.40
XM_927139.1	LOC643869	0.14
XM_933938.2	LOC643872	0.38
XM_928663.1	LOC645649	0.03
XR_037491.1	LOC646808	0.46
XM_943707.1	LOC649431	0.33
XM_941853.1	LOC652416	0.00
XM_928640.1	LOC653651	0.39
XM_001126803.1	LOC728185	0.36
XR_015405.1	LOC728895	0.43
XM_001130993.1	LOC729675	0.23
XM_001714434.1	LOC730376	0.40
NM_002343.2	LTF	0.01
NM_012323.2	MAFF	0.44

NM_005204.2	MAP3K8	0.39
NM_052858.3	MARVELD3	0.33
NM_033290.1	MID1	0.33
NR_030209.1	MIR518E	0.31
NM_173855.3	MORN3	0.40
NM_013404.3	MSLN	0.21
NM_003828.2	MTMR1	0.45
NM_001005474.1	NFKBIZ	0.47
NM_001080379.1	PACRG	0.01
NM_000438.3	PAX3	0.27
NM_003706.1	PLA2G4C	0.41
NM_002658.2	PLAU	0.44
XM_940486.1	PLEKHA2	0.26
NM_014330.2	PPP1R15A	0.46
NM_022114.2	PRDM16	0.11
NM_000963.1	PTGS2	0.32
NM_000963.1	PTGS2	0.40
NM_001024455.2	RGAG4	0.43
NM_184237.1	RNPC2	0.48
NM_001007098.1	SCP2	0.40
NM_000450.1	SELE	0.22
NM_013386.3	SLC25A24	0.41
NR_003237.1	SNORD113-9	0.34
XM_291729.7	TAF3	0.38

NM_031898.1	TEKT3	0.16
NM_001001524.2	TM6SF2	0.10
NM_001097620.1	TMEM184A	0.08
NM_002160.2	TNC	0.45
NM_002160.1	TNC	0.44
NM_006290.2	TNFAIP3	0.28
NM_001561.4	TNFRSF9	0.50
NM_033229.2	TRIM15	0.41
NM_020810.2	TRMT5	0.21
NM_001080419.1	UNK	0.50
NM_030570.2	UPK3B	0.39
NM_001078.2	VCAM1	0.15
NM_206923.1	YY2	0.30
NM_145271.3	ZNF688	0.18

Appendix F. Status of frequently mutated genes identified in CRC cell lines. SNV: Single nucleotide variant; Indel: Insertion/deletion mutations

CRC Cell line	T53	KRAS	BRAF	PIK3CA	PTEN	EGFR	CTNNB1	APC	IKBKB
HT29	p.R273H	WT	p.V600E p.T119S	WT	WT	WT	Copy number loss	SNV/ Indel	Copy number loss
HCT116	WT	p.G13D	WT	p.H1047R	WT	WT	WT	WT	WT
DLD1	p.S241F	p.G13D	WT	p.E545K; p.D549N	WT	SNV/ Indel	WT	SNV/ Indel	WT

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CURRICULUM VITAE

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EDUCATION

2014-2020	Doctor of Philosophy in Pharmacology Indiana University Indianapolis, IN Mentor: Tao Lu, Ph.D.
2003-2007	B.S. Biological Sciences Oakwood University Huntsville, AL

PROFESSIONAL EXPERIENCE

2014-2020	**Graduate Research Assistant** Indiana University School of Medicine, Indianapolis, IN Department of Pharmacology and Toxicology
2010-2013	**Research Specialist** Emory University School of Medicine, Atlanta, GA Center for Neurodegenerative Disease
2007-2010	**Research Assistant** University of Alabama, Huntsville, AL Department of Biology

PEER-REVIEWED PUBLICATIONS

1. **Hartley AV.** et al., & Lu T. Regulation of a PRMT5/NF-κB axis by novel phosphorylation of PRMT5 at serine 15 in colorectal cancer. 2019. *Under revision.*

2. Jin J., Martin M., **Hartley AV**., and Lu T. *PRMTs and miRNAs: functional cooperation in cancer and beyond*. Cell Cycle. 2019; 11 (1): 1538-4101.

3. **Hartley AV,** Martin M, and Lu T. *Epigenetic biomarkers and their therapeutic applications in colorectal cancer.* In: Advances in the molecular understanding of colorectal cancer. 2018. Eva Segelov (Ed.). InTech; ISBN 978-953-51-7585-8.

4. Martin M, **Hartley AV**, Jin J, Sun M, and Lu T. *Phosphorylation of NF-κB in cancer.* In: Adenosine triphosphate in health and disease. 2018. Marcelo L. Larramendy (Ed.). InTech; ISBN 978-953-51-6400-5.

5. **Hartley AV,** Martin M, Jin J, and Lu T. *Epigenetics of aging and cancer: a comprehensive look.* In: Pharmacoepigenetics. 2018. Ramon Cacabelos & Oscar Teijido (Ed.). Elsevier; ISBN 978-012-81-3939-4

6. **Hartley AV**, Wei H, Prabhu L, Martin M, and Lu T. *NF-κB: Its role in colorectal cancer.* In: Transcriptional factors: their role in gastrointestinal malignancies. 2018. Ganji Purnachandra Nagaraju & P. Veera Bramha Chari (Ed.). Springer Nature Singapore; ISBN 978-981-10-6727-3.

7. Martin M, **Hartley AV**, and Lu T. *Colorectal cancer therapeutics: present and future.* In: Cancer Therapeutics. Avid Science. 2018; 2-32. ISBN: 978-93-86337-78-8.

8. Wei H, Prabhu L, **Hartley AV**, Martin M, Sun E, Jiang G, Liu Y, and Lu T. *Methylation of NF-κB and its Role in Gene Regulation.* In: Gene Expression and Regulation in Mammalian Cells - Transcription From General Aspects. 2017. Fumiaki Uchiumi (Ed.). InTech; ISBN 978-953-51-3856-3.

9. **Hartley AV**, Martin M, and Lu T. Aging: Cancer - an unlikely couple. Aging. 2017; 9(9):1949-1950.

10. Martin M, Hua L, Wang B, Wei H, Prabhu L, **Hartley AV**, Jiang G, Liu Y, and Lu T. Novel Serine 176 Phosphorylation of YBX1 Activates NF-κB in Colon Cancer. J Biol Chem. 2017; 292(8):3433-3444.

11. Prabhu L, Mundade R, Wang B, Wei H, **Hartley AV**, Martin M, McElyea K, Temm C, Sandusky G, Liu Y, and Lu T. *Critical role of phosphorylation of serine 165 of YBX1 on the activation of NF-κB in colon cancer.* Oncotarget. 2015; 6(30): 29396-412.

12. Wang B, Wei H, Prabhu L, Zhao W, Martin M, **Hartley AV,** and Lu T. *Role of Novel Serine 316 Phosphorylation of the p65 Subunit of NF-κB in Differential Gene Regulation.* J Biol Chem. 2015; 290(33):20336-47.

13. Prabhu L, **Hartley AV**, Martin M, Warsame F, Sun E, and Lu T. *Role of post-translational modifications of the Y box binding protein 1 in human cancers.* Genes & Diseases. 2015; 2(3):240-46.

14. Huang XP, **Hartley AV**, Yin Y, Herskowitz JH, Lah J, and Ressler K. *AAV2 production with optimized N/P ratio and PEI- mediated transfection results in low toxicity and high titer for in vitro and in vivo applications.* J Virol Methods. 2013; 193(2): 270-277.

PRESENTATIONS

Hartley AV and Lu T. *Regulation of a PRMT5/NF-κB axis by novel serine phosphorylation of PRMT5 in colorectal cancer*. Poster Presentation. Keystone Symposia on Molecular & Cellular Biology (Cancer epigenetics: New mechanisms and therapeutic approaches). 2020 | Keystone, CO, USA.

Hartley AV and Lu T. *Regulation of a PRMT5/NF-κB axis by novel serine phosphorylation of PRMT5 in colorectal cancer*. Poster Presentation. Gordon Research Conference (Epigenetics). 2019 **|** Holderness, NH, USA.

Hartley AV and Lu T. *PRMT5-mediated methylation of YBX1 regulates NF-κB activity in colorectal cancer.* Poster Presentation. American Association for Cancer Research Annual Meeting. 2018 **|** Chicago, IL, USA.

Hartley AV and Lu T. *PRMT5-mediated methylation of YBX1 regulates NF-κB activity in colorectal cancer.* Poster Presentation. IU Simon Cancer Center's Annual Cancer Research Day. 2018 **|** Indianapolis, IN, USA.

Hartley AV and Lu T**.** *What you know can kill you: targeting unique features of cancer cells for drug development.* Oral Presentation. Midwestern Association of Graduate Schools Meeting. Regional Three Minute Thesis Competition. 2018 **|** Grand Rapids, MI, USA.

Hartley AV and Lu T. *Regulation of NF-κB and its role in cancer.* Poster Presentation. IU School of Medicine Annual Poster Showcase Event. Indiana University School of Medicine. 2015-2018 **|** Indianapolis, IN, USA.

Hartley AV and Lu T. *Regulation of PRMT5 by novel serine phosphorylation in colorectal cancer*. Oral Presentation. Department of Pharmacology & Toxicology Student Seminar Series. Indiana University School of Medicine. 2017 & 2018 **|** Indianapolis, IN, USA.

Hartley AV and Lu T**.** *What you know can kill you: targeting unique features of cancer cells for drug development.* Oral Presentation. Preparing Future Faculty & Professionals (PFFP) Conference. IUPUI Three Minute Thesis Competition. 2017 **|** Indianapolis, IN, USA.

Huang XP[1], **Hartley AV[1]**, Yin Y[1], Herskowitz JH[1], Lah JJ[1] and Ressler K[2]. *Simple and effective method for producing high titer recombinant adeno-associated virus 1, 2 and 5 using polyethyleneimine (PEI).* [1] CND, Department of Neurology,[2] General Department of Psychiatry, Emory University, Atlanta, GA 30322. Poster Presentation. 2nd Annual ISBiotech Viral Vector Conference. 2011 **|** Roswell, VA, USA.

HONORS/AWARDS

2019	K.K. Chen Award | Department of Pharmacology & Toxicology
2019	IUPUI Travel Fellowship | IUPUI Graduate School
2019	Paradise Travel Award | Department of Pharmacology & Toxicology, IU School of Medicine
2019	Keystone Symposia Travel Award
2019	Graduate & Professional Education Grant | IUPUI GPSG
2019	Graduate Student Travel Award | IU School of Medicine
2018	Paradise Travel Award | Department of Pharmacology & Toxicology, IU School of Medicine

2018	Institutional Nominee, NIH Pre-doc/Postdoc transition Award (F99/K00) | IU School of Medicine
2018	Scholar-in-Training Award | American Association for Cancer Research
2018	IUPUI Travel Fellowship | IUPUI Graduate School
2018	Dr. Jason T. Spratt Award, Graduate Student Representative of the Year | IUPUI GPSG
2018	Excellence in Basic Science Research Award | IU School of Medicine Diversity Affairs
2018	Graduate Student Volunteer of the Year Award |IU School of Medicine
2017	Winner, Three-minute Thesis Competition | IUPUI Graduate School
2017	Midwestern Association of Graduate Schools Meeting Travel Award | IUPUI
2017	Graduate Student Travel Award |IU School of Medicine
2017	Graduate Women in STEM (GWiSTEM) Fellowship | IU Bloomington

TEACHING & MENTORING

2019	Supervise 2 Ph.D. rotation students Indiana University School of Medicine, Indianapolis IN
2018	Mentor 1 high school student Indiana University School of Medicine, Indianapolis IN
2017	Mentor 3 high school students Indiana University School of Medicine, Indianapolis IN
2012	Train 1 new employee Emory University School of Medicine, Atlanta, GA
2009	Train & Mentor 3 undergraduate students University of Alabama, Huntsville, AL
2007	Microbiology Teaching Assistant Oakwood University, Huntsville, AL

SERVICE

2018-2020	Mentor

	Big Brother Big Sister of Central Indiana
2019	Science Outreach Volunteer Capitol City SDA School
2018-2019	Vice President Graduate & Professional Student Government Indiana University-Purdue University at Indianapolis
2018	Science Outreach Volunteer Unionville Elementary School
2017-2020	Tutor School on Wheels Indianapolis, IN
2017-2018	Graduate Student President & Indiana School of Medicine Representative Department of Pharmacology and Toxicology Indiana University School of Medicine
2017-2018	Member of Graduate Division Travel Grant Review Committee Indiana University School of Medicine Biomedical Gateway program Indiana University School of Medicine
2017-2018	International Graduate Student Welcome Volunteer Office of International Affairs Indiana University-Purdue University at Indianapolis